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Although prostate cancer patients initially respond to androgen ablation therapy, they ultimately relapse and the tumor no longer responds to androgen, offering little hope for long-term disease-free survival. However, inhibition of AR expression in cells leads to cell death. This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen. We have demonstrated that expression of AR in PC3 prostate tumor cells can rescue cells from death induced by inhibition of PI3K. Expression of AR in PC3 cells leads to increased expression of integrin $\alpha 6\beta 1$ and Bcl-xL along with increased activation of NF-$\kappa$B. Blocking each these components individually concurrent with inhibition of PI3K led to death of the AR-expressing cells, suggesting that AR regulates cell survival through enhancement of $\alpha 6\beta 1$/NF-$\kappa$B/Bcl-xL signaling.

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## INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men and death is due to metastasis. While primary prostate tumors are often curable, metastatic tumors are not. Androgen ablation therapy has been the most commonly prescribed treatment for metastatic prostate cancer for the last sixty years. Androgen ablation therapy prevents androgen function by inhibiting both the production of androgen and its binding to its receptor, androgen receptor (AR). Although patients initially respond to treatment, they ultimately relapse and the tumor no longer responds to androgen, offering little hope for long-term disease-free survival. However, inhibition of AR expression or its DNA binding activity even in androgen independent (i.e. non-responsive) cells inhibits their proliferation and leads to cell death. This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen. Thus targeting AR directly or its downstream effectors that regulate survival would be a more effective therapeutic approach for targeting and killing prostate cancer cells. Development of new strategies for more effective treatment of prostate cancer is limited by an incomplete understanding of the mechanisms regulating cell survival of either normal prostate or prostate cancer cells.

Prostate cancer arises from the epithelial layer of the prostate. The normal prostate epithelium consists of two types of cells, basal cells and secretory cells. In the basal cells, which do not express AR, adhesion to the extracellular matrix in the basement membrane is required for cell survival. In the secretory cells, which do express AR, survival is independent of matrix and is suggested to be regulated by AR since these cells die during androgen ablation therapy. In normal prostate epithelial, adhesion to matrix and AR expression are mutually exclusive events. However, in prostate cancer, the tumor cells express AR and are adherent to matrix, allowing for interactions between these two signaling pathways. **My hypothesis was that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while AR regulates survival of normal cells independently of integrins.** The objective of these studies is to identify the AR- and integrin-mediated mechanisms which regulate survival in AR expressing tumor and normal prostate cells. By understanding the activities that lie downstream of AR that directly regulate survival of the tumor cells versus normal cells, a specific approach to disrupt AR-dependent actions only in the tumor cells can be developed, which will lead to the death of tumor cells without harming normal prostate tissues.

## BODY

My working hypothesis is that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while survival of normal cells is regulated independently of integrins. To accomplish the tasks outlined in the statement of work, AR expressing prostate cancer cells and AR expressing normal cells first had to be generated as previously described and validated in the original submission.

### Summary of Aim 1:

The goal of Aim 1 in our Statement of Work was to determine how AR signaling mediates survival in prostate cancer cells *in vitro*. My *working hypothesis* was that AR activation will independently regulate the same downstream survival targets as those regulated by the PI3K/Akt pathway, such as survivin, such that inhibition of signaling from either PI3K/Akt or AR can be rescued by the other pathway**.** I proposed to have the tasks in Aim 1 completed in the first year of funding. **Task A** was to determine if AR expression affects integrin-mediated survival signaling pathways in DU145s. I along with another graduate student, Jelani Zarif, have begun these studies. Similar to what we found in PC3 cells (2008 report), we have found that integrin α6 expression is up-regulated in AR expressing DU145 cells (data not shown). **Task B** was to determine if LM signaling via AR to survivin rescues survival in PI3K inhibited

cells. Emphasis was placed on the **Task B** and Aim 2, and given time constraints, this leaves **Task A** still needing to be accomplished. However, the **Task B** is complete. As of last year's report, I had demonstrated that AR can promote prostate tumor cell survival independent of the PI3K pathway. AR did not affect survivin expression. AR regulated integrin expression by decreasing integrins α3, α5, β1, and β4 and increasing integrin α6. Up-regulation of integrin α6 drove increased expression of the pro-survival protein Bcl-xL. Bcl-xL promotes survival independent of PI3K signaling. AR also enhanced Src activity, independent of integrin α6. Enhanced Src activity was not responsible for elevated Bcl-xL levels. We are currently preparing a manuscript for submission to a peer-reviewed journal describing the results I have obtained thus far and have described below.

Viruses were used to introduce empty vectors (PC3-puro or pLKO), sequence-verified wild-type AR, or two AR mutants into PC3 cells. These cell lines were selected and constantly maintained in charcoal-stripped serum to avoid selection against growth suppression, a problematic side-effect of AR expression in PC3 cells (1). AR expression was constantly monitored and only early-passage (< 20) cells were used. To understand which function of AR is important to the observed phenotypes, two well-characterized AR mutants were selected. The ΔNLS mutant is defective in AR translocation to the nucleus and in DNA binding (2, 3). The ΔLBD mutant is unable to bind ligand (4). To ensure that AR was not too highly over expressed, stable clonal cell lines that expressed approximately the same level of AR as LNCaP cells were selected. AR expression, in the absence of androgens, was confirmed by immunoblotting and immunoflourescent (IF) staining with anti-AR antibodies (Fig. 1A, B). All the AR mutants had approximately the same level of AR expression as the LNCaP cells (Fig. 1A). AR localization was both cytoplasmic and nuclear in wild-type AR expressing clones by IF staining (Fig. 1B). Surprisingly, the ΔLBD mutant was predominately nuclear, while the ΔNLS mutant was exclusively cytoplasmic as expected (Fig. 1B). AR localization was not significantly altered by exogenous treatment with androgens (DHT). Inhibition of PI3K signaling in PC3 cells with the pharmalogical inhibitor LY294002 results in cell death (5). In contrast, the AR-expressing clones did not experience significant cell death from LY294002 treatment on LM1 (2008 report). This difference in survival was not strictly due to cell cycle status since PC3-AR-1 cells grow at the same rate, while PC3-AR-2 cells grow slower than PC3-puro cells (2008 report). Furthermore, the ability of AR-expressing cells to survive on LM1 was not due to AR-mediated hyper-activation of the PI3K/Akt pathway and LY294002 was still a potent inhibitor of PI3K/Akt signaling in the AR expressing cells (2008 report). The AR nuclear localization mutant ΔNLS (Fig. 1C), but not the AR ligand binding mutant ΔLBD (Fig. 1D), restored sensitivity to PI3K inhibition resulting in cell death. Thus, AR can promote survival independent of PI3K signaling in PC3 cells plated on LM1. This requires AR localization to the nucleus, presumably to bind DNA.

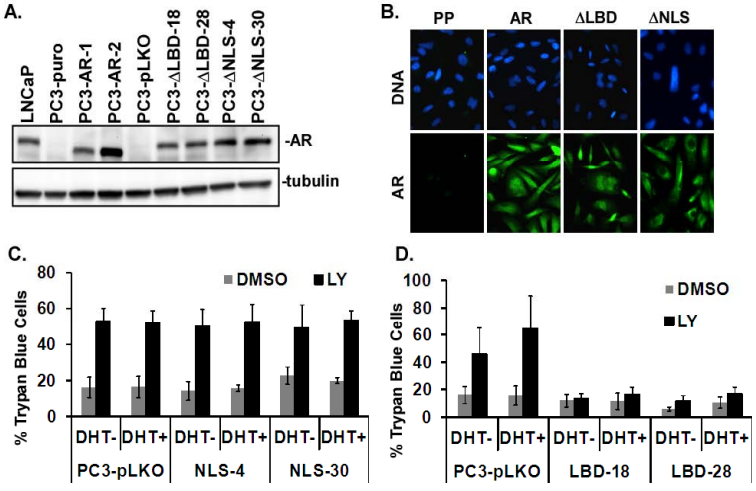


**FIGURE 1. Nuclear Localization, but not ligand binding, required for AR-mediated survival. A)** AR and tubulin expression by immunoblotting of LNCaP, PC3 empty-vector (PC3-puro or PC3-pLKO) pools and AR expressing clones (PC3-AR, PC3-ΔLBD, and PC3-ΔNLS). **B)** PC3-puro (PP), PC3-AR-1 (AR), PC3-ΔLBD-28 (ΔLBD), and PC3-ΔNLS-4 (ΔNLS) cells were immunostained for AR (green) and DNA (blue). **C-D)** PC3-pLKO, PC3-ΔNLS (NLS), or PC3-ΔLBD (LBD) cells were growth factor-starved in charcoal-stripped media, and plated on LMI with vehicle or 10 nM DHT in the presence of DMSO or 10 μM LY294002 (LY). After 72 hrs cells were counted for trypan blue staining**.** Error bars on all graphs represent standard deviation; *n* = 3-5.

AR is a transcription factor whose activity depends on nuclear localization. Expression of the AR ΔNLS mutant that is unable to translocate into the nucleus and bind DNA, was also unable to protect cells from LY294002-induced death (Fig 1 C,D). To determine if AR expression was regulating integrin α6 and Bcl-xL transcription, RNA was isolated from PC3-puro and AR expressing clones, reverse transcribed and quantitative RT-PCR (qRT-PCR) was preformed. There was over a 10-fold increase in integrin α6 and Bcl-xL mRNA levels compared to PC3-puro cells, independent of DHT addition (Fig. 2A,B). The AR increase in Bcl-xL mRNA is in agreement with studies in LNCaP cells where treatment with androgen or AR-specific siRNA leads to a respective increase or decrease in Bcl-xL mRNA (6, 7). To verify that this was an AR-dependent effect, PC3-AR-1 cells were treated with the anti-androgen RU486 or AR-specific siRNA. This was done in collaboration with another graduate student, Jelani Zarif. RU486 is reported to recruit co-repressors to AR transcriptional complexes thereby inhibiting AR-mediated transcription (8). RU486 decreased integrin α6 mRNA expression as measured by RT-PCR, (Fig. 2C). RU486 treatment also resulted in a decrease in the protein levels of integrin α6 expression with approximately the same severity as AR-specific siRNA treatment (Fig. 2D). There was also a decrease in integrin β1 (Fig. 2D).

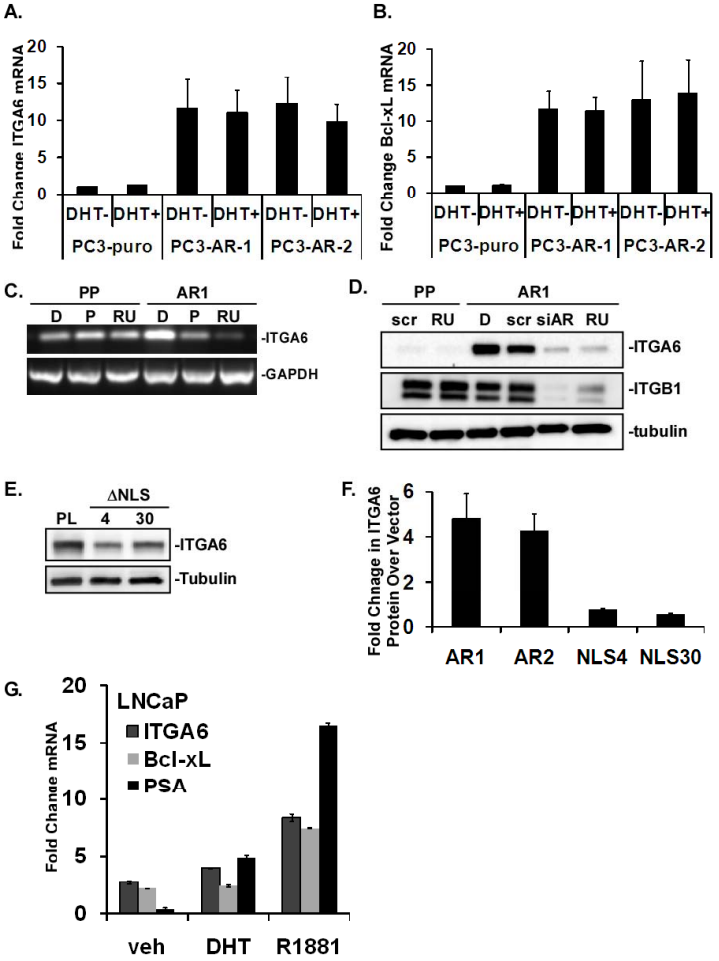


**FIGURE 2. AR regulates integrin α6 and Bcl-xL mRNA expression.** C3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 (AR2) cells were plated on LMI and treated with vehicle (ethanol) or DHT for 72 hours. Integrin α6 (ITGA6) **A)** or Bcl-xL **B)** mRNA expression was measured by qRT-PCR. Gene expression was normalized to 18s rRNA then expressed as fold change relative to vehicle-treated PC3-puro cells. Error bars represent standard deviation; *n* = 3. **C)** Cells were plated on LM1 then treated with DMSO (D), PBS (P), or RU486 (RU) for 72 hours. Integrin α6 (ITGA6) and GAPDH mRNA expression was analyzed using RT-PCR. **D)** Cells were treated with DMSO (D), RU486 (RU), AR siRNA (siAR), or non-targeting siRNA (scr) for 72 hours. Cells were lysed and immunobloted to monitor integrin α6 (ITGA6), β1 (ITGB1), and tubulin expression. **E)** PC3-pLKO (PL) and PC3-ΔNLS-AR (ΔNLS) clone lysates were monitored for integrin α6 (ITGA6) and tubulin expression by immunoblotting. **F)** Cells were growth factor-starved in charcoal-stripped media and plated on LMI. After 72 hours, cells were treated with flourescent-conjugated integrin α6 antibody and analyzed by FACS. Rat IgG controls were subtracted from mean flourescent values then values for AR expressing cells were normalized to those of the corresponding vector cells. Error bars represent standard error; *n* = 2. **G)** LNCaP cells were serum- starved in charcoal-stripped media for 48 hours, then treated with vehicle (veh), DHT, or R1881 for 24 hours. Integrin α6 (ITGA6), Bcl-xL, and PSA mRNA expression was measured by qRT-PCR. Gene expression was normalized to 18s rRNA then expressed as fold change relative to untreated cells. Error bars represent standard deviation; *n* = 1. *(A-B, E-G) Laura Lamb, (C-D) Jelani Zarif.*

Since integrins must be expressed as heterodimers in order to be stably expressed, loss of the integrin α6 binding partner of integrin β1 may be leading to its degradation. Since AR

must be in the nucleus to act as a transcription factor, we tested the affect of expression of the AR ΔNLS mutant on integrin α6 and Bcl-xL expression. Expression of the ΔNLS AR mutant in PC3 cells did not result in increased integrin α6 expression compared to empty vector PC3-pLKO cells (Fig. 2 E,F). Lastly, stimulation of LNCaP cells for as little as 24 hours with DHT or the more potent synthetic androgen R1881 results in increased integrin α6 and Bcl-xL mRNA expression as determined by qRT-PCR. PSA was used as a positive control. Together, this suggests that AR transcriptionally regulates integrin α6 and Bcl-xL expression.

The effect of AR on Bcl-xL expression is indirect, since we demonstrated that α6 is required for Bcl-xL expression (2008 Report). We sought to determine how integrin α6 could drive Bcl-xL expression, since it was not through Src (2008 Report). The transcription factor NF-κB (RelA) has been reported to directly bind the promoter of and drive transcription of Bcl-xL (9, 10). Furthermore, increased NF-κB activity is associated with prostate cancer progression (11), castration-resistance (12, 13), poor prognosis (14, 15), biochemical failure (i.e. PSA relapse) (16, 17), and has been determined to be significantly misregulated in metastatic prostate cancer based on microarray studies (18). Therefore, we next determined if NF-κB signaling was increased in AR-expressing cells. NF-κB p65 activity, as determined by both NF-κB p65 phosphorylation and a reporter assay, were increased in AR-expressing cells (Fig. 3A,B). This is in agreement with previous work that expression of AR in PC3 cells can result in increased NF-κB activity (19). The increase in NF-κB reporter activity was also independent of DHT (Fig. 3B). To determine if AR and integrin α6 regulated NF-κB signaling, AR and integrin α6 were knocked down in AR expressing cells using siRNA. Knock-down of AR or integrin α6 resulted in a decrease in NF-κB p65 phosphorylation and in some cases, a modest decrease in total NF-κB protein levels (Fig. 3C,D). TNFα stimulation of PC3-puro cells was used as a positive control for NF-κB p65 phosphorylation (Fig. 3C,D). Thus, NF-κB p65 activity was increased in AR expressing cells in an AR- and integrin α6-dependent manner.

To determine if NF-κB p65 (RelA) was regulating Bcl-xL expression, AR expressing cells were treated with NF-κB p65 specific siRNA. Knock-down of NF-κB p65 in AR expressing cells resulted in only a partial knock-down of Bcl-xL (Fig. 3E), suggesting that another pathway or another NF-κB family member may also be important in regulating Bcl-xL expression in AR expressing cells. Indeed, AR has been reported to directly regulate Bcl-xL expression (7), which could explain why NF-κB p65 knock-down only resulted in partial loss of Bcl-xL. Knock-down of NF-κB did not alter integrin α6 expression (data not shown). Nonetheless, knock-down of NF-κB p65 was sufficient to sensitize AR-expressing cells to LY294002-induced death (Fig. 3F). Similar results were seen using the cell permeable small peptide inhibitor SN50, which blocks NF-κB translocation into the nucleus (data not shown). Thus, the partial knock-down of Bcl-xL by NF-κB loss may be sufficient for AR expressing cells to regain dependence on PI3K signaling. However, NF-κB signaling is known to regulate other key regulators of cell survival, including cFLIP, BFl-1/A1, c-IAP1/2, and XIAP (20), whose loss in expression may also contribute to this phenotype.

Classically NF-κB is kept in the cytoplasm bound to a family of inhibitor proteins, called IκBs (inhibitor of κB), where IκBα is the most prevalent and characterized isoform (21, 22). Activation of NF-κB classically requires phosphorylation and subsequent degradation of IκB, which allows NF-κB to translocate into the nucleus (21, 22). Surprisingly, we did not observe any detectable phosphorylation or loss of IκBα in our AR expressing cells compared to the PC3-puro cells (Fig. 3G). TNFα stimulation of PC3-puro cells was used as a positive control for phosphorylation of IκBα (Fig. 3G). Knock-down of AR or integrin α6 also did not have an effect on IκBα phosphorylation or degradation (Fig. 3G, data not shown). IκBα is phosphorylated by IκB kinase (IKK) (21, 22). PC3 cells are reported to have constitutively active IKKα, resulting in enhanced IκBα degradation and constitutive active NF-κB activity (19, 23). IKK activity as

measured by phosphorylation was also not detectable in our cells by immunoblotting, although stimulation with TNFα did not result in robust detection of phosphorylation of IKKα/β by immunoblotting either (data not shown). Therefore, we cannot yet determine if NF-κB is being activated through the canonical or a non-canonical pathway.

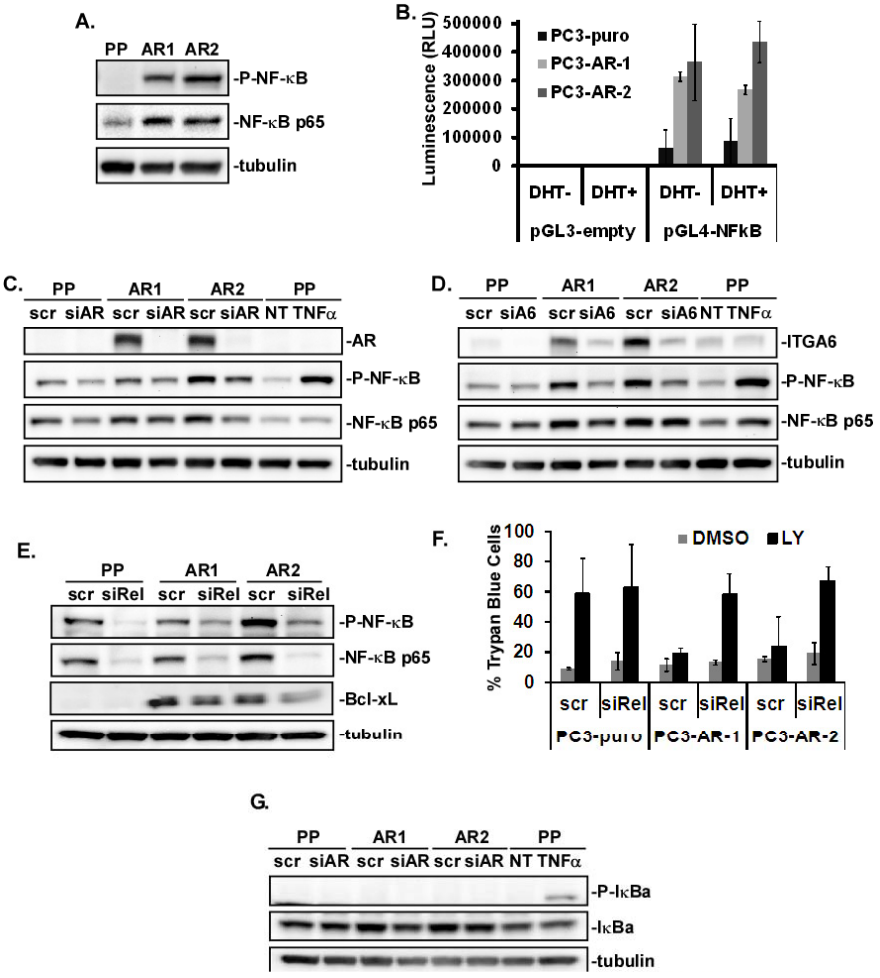


**FIGURE 3. AR and integrin α6 regulate NF-κB signaling.** PC3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 cells were serum starved in charcoal stripped serum and plated on LMI. **A)** Cells were lysed to monitor RelA phosphorylation (P-NF-κB) and total levels of RelA (NF-κB p65). Tubulin expression was used as a loading control. **B)** Cells were transfected with empty (pGL3-empty) or NF-κB expressing Firefly reporter (pGL4-NFκB) vector and control *Renilla* reporter then treated with vehicle or DHT for 72 hours. Cells were then lysed and luminescence measured. Firefly luminescence activity was normalized to *Renilla* luciferase activity. **C-F)** Cells were treated with siRNA against AR (siAR), integrin α6 (siA6), RelA (siRel), or non-targeting sequence (scr) for 72 hours, and in some cases not treated (NT) or treated with 10 ng/mL TNFα for 1 hours. **C-E)** Cells were then lysed to monitor RelA phosphorylation (P-NF-κB) and total levels of RelA (NF-κB p65), AR, integrin α6 (ITGA6), or Bcl-xL. Tubulin expression was used as a loading control. **F)** Cells were plated on LMI and treated with DMSO or LY294002 (LY) for 72 hours. Cell viability was then measured by trypan blue staining. Error bars represent standard deviation; *n* = 3. **G)** Cells were treated with siRNA against AR (siAR) or non-targeting sequence (scr) for 72 hours, and in some cases not treated (NT) or treated with 10 ng/mL TNFα for 1 hours. Cells were then lysed to monitor IκBa phosphorylation (P-IκBa) and total levels of IκBa. Tubulin expression was used as a loading control.

In MCF10A cells, integrin α6 promotes survival by p21-activated kinases 1 (PAK1) activation of NF-κB p65 (24). Others have also shown that multiple signaling pathways can activate NF-κB via PAK1 (25). Thus, we investigated if integrin α6 up-regulates NF-κB expression via activation of PAK1. PAK1 activity was measured using a phospho-specific antibody that recognizes the active form of PAK1/2. PAK1/2 phosphorylation and activity was increased in both AR clones compared to PC3-puro cells (Fig. 4A). Surprisingly, there was decreased expression of PAK1 in AR expressing cells compared to PC3-puro cells, although levels of PAK2 were relatively unchanged (Fig. 4A). PAK3 was undetected by immunoblotting (data not shown). To determine if PAK signaling was regulated by AR and integrin α6, AR expressing cells were treated with AR and integrin α6 specific siRNA, with non-specific siRNA (scram) being used as a negative control. Knock-down of AR led to a decrease in PAK1/2

phosphorylation, (Fig. 4B). Knock-down of integrin α6 in AR expressing clones to PC3-puro levels resulted in a more dramatic loss in PAK1/2 phosphorylation (Fig. 4C). Thus, there is an increase in PAK1/2 activity in AR expressing cells that is both AR and integrin α6 dependent.

Since the phosphorylation-specific antibody recognizes both PAK1 and PAK2, it is difficult to determine which isoform, or if both, may be active. However, since there are low levels of PAK1 in the AR expressing cells, it is likely PAK2 is the predominant active isoform in the AR expressing cells. Immunoprecipitation of PAK1 and immunoblotting with the phospho-specific antibody of these cells suggested that PAK1 was poorly phosphorylated, further supporting that PAK2 is more active (not shown). It has also been reported that PAK2 can inhibit PAK1 activity in PC3 cells (26). If this is the case, then loss of AR or integrin α6 activation of PAK2 should lead to an increase in PAK1. Knock-down of AR in PC3-AR-1 cells, but not PC3-puro cells or PC3-AR-2 cells, results in increased PAK1 expression (Fig. 4B). Loss of integrin α6 in all cell lines resulted in increased PAK1 expression (Fig. 4C). Together, this suggests that there is increased PAK activity, probably through PAK2, in AR expressing cells that is both AR- and integrin α6-dependent.

To determine if PAK signaling regulates NF-κB p65 and Bcl-xL, the cells were treated with the group I specific PAK pharmalogical inhibitor, IPA-3 (27). IPA-3 inhibits PAK1-3, with the strongest inhibition observed for PAK1 (27). Treatment of AR expressing cells with IPA-3 resulted in decreased NF-κB phosphorylation and decreased Bcl-xL expression (Fig. 4D). IPA-3 treatment has no effect on AR or integrin α6 expression (data not shown). In an additional approach, AR expressing cells were treated with siRNA specific to PAK1. Knock-down of PAK1 was over 90% in AR expressing cells and did not affect PAK2 expression (Fig. 4E). Knock-down of PAK1 resulted in only partial loss of PAK1/2 and NF-κB p65 phosphorylation in AR expressing cells (Fig. 4E). There was a very modest decrease in total NF-κB p65 levels, and a modest decrease in Bcl-xL expression (Fig. 4E). While this suggests that PAK1 may be upstream of NF-κB signaling and Bcl-xL expression, it may not be the only protein regulating this pathway since PAK1 specific knockdown did not result in a dramatic decrease in NF-κB activity or Bcl-xL expression, as was observed with AR or integrin α6 knockdown, or treatment with the group I PAK inhibitor, IPA-3 (Fig. 3C,D; Fig. 4D,E).

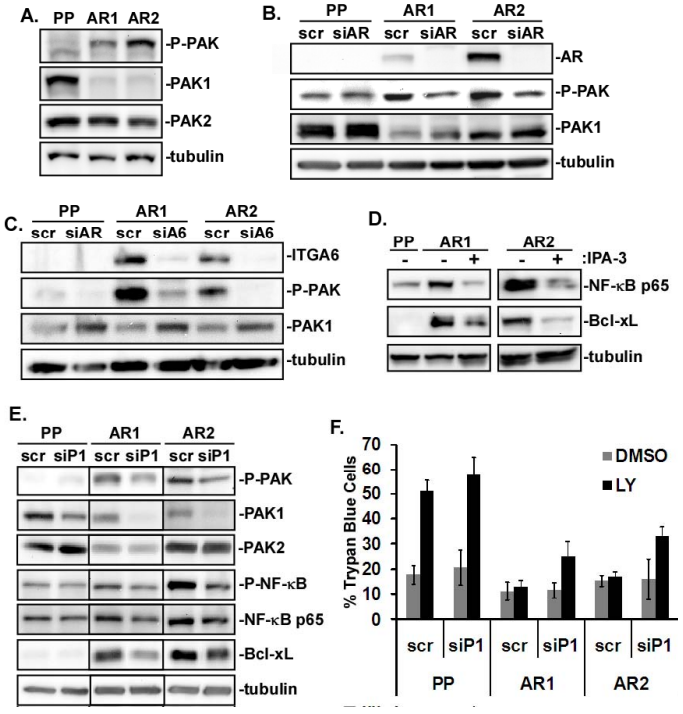


**FIGURE 4. AR and integrin α6 regulate PAK1/2 signaling.** PC3-puro (PP), PC3-AR-1 (AR1), and PC3-AR-2 cells were serum starved in charcoal stripped serum and plated on LMI. **A)** Cells were lysed to monitor PAK1/2 phosphorylation (P-PAK), PAK1 and PAK2, and tubulin expression. **B-C)** Cells were treated with siRNA against AR (siAR), integrin α6 (siA6), or non-targeting sequence (scr) for 72 hours, then lysed to monitor PAK1/2 phosphorylation (P-PAK) and expression of AR, integrin α6 (ITGA6), PAK1, PAK2, and tubulin. **D)** Cells were treated with vehicle or 30 μM IPA-3 for 1 hour then lysed and immunoblotted to monitor total levels of RelA (NF-κB p65) and Bcl-xL. Tubulin expression was used as a loading control. **E-F)** Cells were treated with siRNA against PAK1 (siP1) or non-targeting sequence (scr) for 72 hours. **E)** Cells were then lysed and immunoblotted to monitor phosphorylation of PAK1/2 (P-PAK) and RelA (P-NF-κB) as well as PAK1, RelA (NF-κB p65), and Bcl-xL expression. Total loading levels were monitored by probing for tubulin. **F)** Cells were plated on LMI and treated with DMSO or LY294002 (LY) for 72 hours. Cell viability was then measured by trypan blue staining. Error bars represent standard deviation; *n* = 3.

PAK1 and PAK2 have some redundant functions; it may be that PAK2 is the predominant PAK as suggested earlier, or that both PAK1 and PAK2 are both required in this pathway. IKKB or p38 has also been reported to phosphorylate NF-κB p65 (28-30). In agreement with this, knock-down of PAK1 alone using siRNA is unable to restore LY294002 sensitivity to death significantly in AR expressing cells (Fig. 4F). PAK4 has also been demonstrated to activate NF-κB signaling (31). Therefore, PAK1 is activated by AR and integrin α6, but there may be redundancy with other PAKs. Other pathways in addition to PAK1 may also responsible for Bcl-xL regulation.

In **summary** of Aim 1, we demonstrated that re-expression of wild type AR in PC3 cells prevented the cell death normally induced upon inhibition of PI3K signaling, independent of androgen. Re-expression of AR in PC3 cells lead to increased expression of integrin α6 and subsequent activation of PAK and NF-κB and increased expression of the pro-survival protein Bcl-xL (Fig. 5). Loss of AR, integrin α6, NF-κB, or Bcl-xL re-sensitized AR-expressing PC3 cells to PI3K-dependent survival. Treatment of AR expressing PC3 cells with the AR inhibitor RU486 or AR specific siRNA, or expression of AR mutants lacking the ability to translocate to the nucleus (ΔNLS), but not to bind ligand (ΔLBD), largely restored the parental PC3 phenotype, including PI3K dependent survival. These results are supported by siRNA knock-down of endogenous AR in LNCaP cells. Thus AR can support castration-resistant prostate tumor cell survival on laminin via enhanced expression of α6β1 integrin, leading to elevated Bcl-xL levels, by a mechanism that is independent of PI3K.

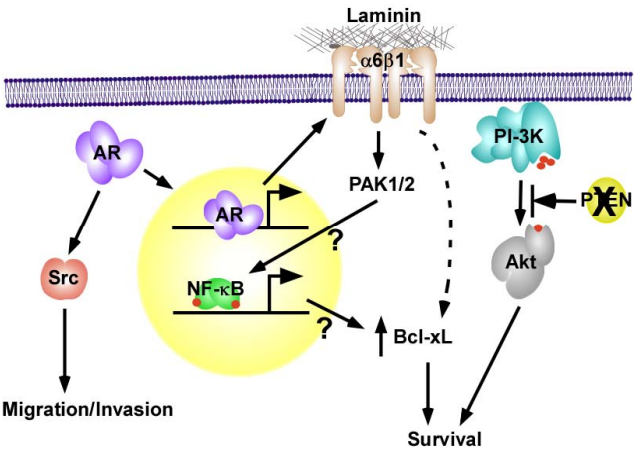


**FIGURE 5. Model for AR signaling in PC3 cells.** AR promotes cell survival independent of DHT or PI3K signaling. AR regulates survival via the integrin α6, which leads to phosphorylation of PAK1/2 and subsequent up-regulation of NF-κB signaling. This may lead to increased Bcl-xL expression, which regulates survival independent of the PI3K pathway. AR increases Src activity independent of integrin α6. Src activity may regulate migration and invasion.

**Summary of Aim 2:**

The goal of Aim 2 is to determine how AR mediates survival in normal primary prostate epithelial cells *in vitro.* My *working hypothesis* was that the integrin-mediated survival pathway in primary prostate epithelial cells will shift from being dependent on EGFR to being dependent on AR. In addition, AR regulates survival by directly regulating survivin. **Task A** was to determine whether AR expression affects integrin-mediated survival signaling pathways in PECs. **Task B** was to determine if integrins mediate survival in PECs expressing AR. **Task C** was to determine if E-cadherin signaling to PI3K and Src mediate survival in PECs expressing AR. **Task D** was to determine if autophagy is mediating survival in PECs expressing AR. I had proposed to complete the tasks in the second to third year of funding. However, I have made significant progress on Aim 2 in the first two years of funding, completing the first two tasks and most of the third (2008 Report). These results have been published in the below reference (32) that has also been attached as a pdf.

Lamb, L.E., Knudsen, B.S., and Miranti, C.K., *E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model.* J. Cell Science, 2010. **123**: p. 266-276

**Task C** was to determine if E-cadherin signaling to PI3K and Src mediate survival in PECs expressing AR. I have determined that E-cadherin signaling to PI3K does mediate

survival in PECs expressing AR. I still need to determine if Src is required for cell survival. To determine if Src is promoting cell survival, differentiated AR-expressing cells will be treated with siRNA specific to Src or with a non-targeting siRNA control. Cell viability will be assessed over a 96 hour time course by TUNEL staining. To determine if E-cadherin signaling is activating Src, differentiated cells will be treated with an E-cadherin blocking antibody or IgG control. The top cells will then be separated from the bottom cells as previously described (32). Src is activated by phosphorylation at Y416 and inhibited by phosphorylation at Y527 (33). Therefore, Src expression and activity was measured in both cell populations by immunoprecipitation of Src and immunoblotting for phosphorylation of Y416, dephosphorylation of Y527, and total Src and Src activity will be monitored by immunoprecipitation of Src.

**Task D** is to determine if autophagy is mediating survival in PECs expressing AR. To determine if autophagy is occurring in the AR positive upper layer, differentiated cells will be starved of growth factors and nutrients over a daily time course up to 96 hours. LC3 protein is generally present throughout the cell, and upon induction of autophagy it is processed and incorporated into autophagic vacuoles. Induction of autophagy is indicated by a shift from very diffuse LC3-GFP fluorescence throughout the cell to punctuate fluorescence within the cytoplasm (34). Autophagy will be monitored by two approaches. The first approach will be to monitor for the presence of LC3-GFP puncta when the differentiated cultures are were infected with an adenovirus that expresses an LC3-GFP fusion protein. For the second approach, the top cells will then be separated from the bottom cells as previously described (32), and autophagy will be monitored by immunoblotting for LC3 processing. To determine if autophagy is required for survival under starvation conditions, autophagy will be inhibited by the lysosomal inhibitor chloroquine, or the Vps34 inhibitor 3-methyladenine. The viability of the AR positive PECs in the upper layer will be assessed by TUNEL staining and confocal microscopy.

**KEY RESEARCH ACCOMPLISHMENTS**

1. Generated the new cell lines/models PC3-AR, PC3-ΔNLS-AR, and PC3-ΔLBD-AR.
2. Determined that nuclear localization, but not ligand binding, is required for AR-mediated survival.
3. Determined that AR regulates integrin α6 and Bcl-xL mRNA expression.
4. Demonstrated that expression of AR in PC3 cells up-regulated PAK and NF-κB activity.
5. Determined that expression of AR in PC3 cells regulates survival via NF-κB and Bcl-xL.
6. Demonstrated that PAK1 does not regulate survival in AR expressing PC3 cells.
7. Generated a new model for AR-expressing, secretory-like, normal prostate cells.

**REPORTABLE OUTCOMES**

The following items have been generated due to the research carried out in the last year.

1. In the past year, three abstracts were presented at scientific meetings as poster presentations. A copy of one of the abstracts is included in the appendix.

   **Lamb, L.E**., Zarif, J.C., Miranti, C.K. 2009. AR-Enhanced α6β1 Integrin and Bcl-$x_L$ Expression Promotes Androgen-Independent Prostate Tumor Cell Survival Independently of PI3K Signaling**.** American Association for Cancer Research: "Advances in Prostate Cancer Research", San Diego, CA, January 21-24.

   **Lamb, L.E.**, Knudsen, B.S., Miranti, C.K. 2009. E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model. Michigan Prostate Research Colloquium: "Prostate Tumor Microenviroment and Metastasis", Detroit, MI, May 30.
   Also won a Third Place Poster Award.

**Lamb, L.E.**, Knudsen, B.S., Miranti, C.K. 2009. A Novel *In Vitro* Differentiation Model to Study Survival Signaling of Androgen Receptor Expressing Prostate Secretory Cells**.** American Association for Cancer Research: "Advances in Prostate Cancer Research", San Diego, CA, January 21-24.

2. In the past year, I presented an oral presentation at a scientific meeting. The abstract is included in the appendix.
   **Lamb, L.E.**, Knudsen, B.S., Miranti, C.K. 2009. E-Cadherin-mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified *In Vitro* Differentiation Model. 2nd Salk Institute Mechanisms and Models of Cancer meeting, La Jolla, CA, August 12-16.

3. I published the following paper.
   **Lamb, L.E**., Knudsen, B.S., and Miranti, C.K., *E-Cadherin-Mediated Survival of Androgen Receptor Expressing Secretory Prostate Epithelial Cells Derived from a Stratified In Vitro Differentiation Model.* J. Cell Science, 2010. **123**: p. 266-276.

4. I co-filed a patent based on the newly published work.
   A Method for Inducing Differentiation of Androgen Receptor-Expressing Prostate Epithelial Cells
   U.S. Patent Office Application No. 61173783, April 29, 2009.

5. I joined two professional societies, Alpha Epsilon Delta and Sigma Xi (MSU Chapters).

6. I will graduate from Michigan State University with my PhD this Spring (2010).

**CONCLUSIONS**

These studies have demonstrated that expression of AR in PC3 cells can rescue cells from death induced by inhibition of PI3K when adherent to laminin 1. Expression of AR in PC3 cells leads to increased expression of integrin α6β1 and Bcl-xL along with increased activation of NF-κB. Blocking each of these components individually concurrent with inhibition of PI3K led to death of the AR-expressing cells, suggesting that AR regulates cell survival through enhancement of α6β1/NF-κB/Bcl-xL signaling. To assess the role of AR in normal cell survival, we generated an in vitro differentiation model. Confluent primary human prostate epithelial cell cultures were treated with KGF and androgen (DHT). After two weeks, a suprabasal cell layer formed in which cells no longer expressed integrins, p63, K5/14, EGFR, FGFR2IIIb, or Bcl-2, but instead expressed AR and androgen-induced differentiation markers, including K18/19, TMPRSS2, Nkx3.1, PSMA, KLK2 and secreted PSA. Differentiated prostate cell survival depended on E-cadherin and PI3K, but not KGF, DHT, AR or MAPK. Therefore, while in the prostate tumor cell line PC3, AR and integrin α6β1 cooperate to drive cell survival, neither AR nor integrins were required for survival of differentiated prostate epithelial cells.

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## APPENDIX

## ABSTRACTS

**Poster Presentation:**
**American Association for Cancer Research: "Advances in Prostate Cancer Research"**, San Diego, CA, January 21-24.

### AR-Enhanced α6β1 Integrin and Bcl-$x_L$ Expression Promotes Androgen-Independent Prostate Tumor Cell Survival Independently of PI-3K Signaling

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Background: Although prostate cancer patients initially respond to androgen ablation therapy, they ultimately relapse and the tumor no longer responds to androgen, offering little hope for long-term disease-free survival. However, inhibition of AR expression or its DNA binding activity even in androgen independent (i.e. non-responsive) cells inhibits their proliferation and leads to cell death. This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen.

Objective: In prostate cancer, unlike in the normal gland, the tumor epithelial cells expressing AR are adherent to extracellular matrix, allowing for potential interactions between integrins, involved in cell matrix adhesion, and AR. Signaling pathways stimulated by both AR and integrins are known to regulate cell survival. **We tested the hypothesis that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR cooperatively regulate their survival.**

Methods: Androgen receptor expression was restored to near physiological levels in PC3 cells. The signaling pathways that are required for survival of AR-expressing PC3 cells plated on the extracellular matrix laminin were compared to those required for non-AR expressing cells.

Results: Survival of PC3 cells adherent to laminin is dependent on PI-3K signaling. Re-expression of wild type AR in PC3 cells prevented the cell death normally induced upon inhibition of PI-3K signaling. Rescue of cell death occurred independently of androgen. Expression of AR in PC3 cells lead to increased expression of the pro-survival protein Bcl-$x_L$ and α6β1integrin, and down regulation of other integrins. Loss of AR, integrin α6, or Bcl-$x_L$ re-sensitized AR-expressing PC3 cells to PI-3K-dependent survival. The AR-induced increase in α6 integrin is responsible for the elevated Bcl-$x_L$ levels. Thus AR regulates cell survival through enhancement of α6β1 expression, which up-regulates Bcl-$x_L$, independently of PI-3K signaling. We are currently exploring the mechanism by which AR enhances α6β1 expression.

Conclusions: AR can support androgen-independent prostate tumor cell survival on laminin via enhanced expression of α6β1 integrin, leading to elevated Bcl-$x_L$ levels, by a mechanism that is independent of PI-3K. These findings have significant implications for therapeutic targeting of androgen-independent prostate cancer cells.

**Oral Presentation:2nd Salk Institute Mechanisms and Models of Cancer meeting**, La Jolla, CA, August 12-16.

**E-Cadherin-mediated survival of Androgen Receptor expressing secretory prostate epithelial cells derived from a stratified in vitro differentiation model**

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Development of new strategies for more effective treatment of prostate cancer is limited by an incomplete understanding of the mechanisms regulating survival of not only prostate cancer cells, but normal prostate cells as well. Prostate cancer arises from the androgen receptor (AR) expressing differentiated secretory epithelial cells of the prostate. However, it is unclear whether androgens directly promote the survival of secretory cells, or whether secretory cells survive through androgen dependent signals from the prostate stroma. Biochemical and mechanistic studies have been hampered by inadequate cell culture models. In particular, large-scale differentiation of prostate epithelial cells in culture has been difficult to achieve. Here we describe the development of a differentiation system that is amenable to functional and biochemical analysis and its application to deciphering the survival pathways in differentiated AR-expressing epithelial cells. Confluent prostate epithelial cell cultures were treated with keratinocyte growth factor (KGF) and DHT. After two weeks, a suprabasal cell layer formed in which cells no longer expressed integrins, p63, K5/14, EGFR, KGFR2IIIb, or Bcl-2, but instead expressed AR and androgen-induced differentiation markers, including K18/19, TMPRSS2, Nkx3.1, PMSA, KLK2, and secreted prostate specific antigen (PSA). Differentiated prostate cell survival depended on E-cadherin and PI-3kinase, but not KGF, androgen, AR or MAPK. Thus survival of differentiated prostate epithelial cells is mediated by cell-cell adhesion, and not through androgen activity or prostate stroma-derived KGF. This is in stark contrast to previous work from our laboratory which demonstrated that integrin-mediated survival of primary prostate basal cells requires integrin-induced EGFR signaling to Erk, but not PI-3K. Thus, there is switch from Erk- to PI-3K-dependent survival in the secretory-like cells. In prostate cancer, there is a strong dependence on PI-3K signaling for survival, as these cells tend to acquire mutations in Pten. This suggests that prostate cancer may arise from a more differentiated cell that has already acquired dependence on PI-3K for its survival, which may be a transformed derivative of the AR expressing secretory epithelial cells of the prostate. We propose that this system can be used to test this hypothesis and better understand prostate cancer development and progression in which immortalization or genetic mutations associated with prostate cancer progression can be tested to see which molecular events are sufficient to switch cells to a prostate tumor phenotype. This knowledge will be critical for understanding prostate cancer progression.

**REVISED STATEMENT OF WORK (SOW):**

The overall objective of this proposal is to determine the mechanism by which androgen receptor (AR) and integrins regulate survival in prostate tumor cells and AR expressing normal prostate cells. To achieve this objective we will carry out the tasks outlined below.

**AIM 1: Determine how AR signaling mediates survival in prostate cancer cells *in vitro*.**
My *working hypothesis* is that AR will promote survival independently of the PI-3K/Akt pathway via the pro-survival protein, Bcl-xL.

**Task A: Determine if AR expression affects integrin-mediated survival signaling pathways in DU145s.** (Months 1-4).

1. DU145 and DU145 AR-expressing cells will be treated with specific pharmacological inhibitors PD168393 or AG1475, PD98059 or U0126, LY294002, and Src specific siRNA.
2. Cells will be plated on CL or LM and cell viability will be analyzed by TUNEL staining and quantified by FACS at daily time points up to 96 hours.

Outcome: It will be determined if EGFR/Erk, PI-3K, and Src are required for integrin-mediated survival pathways in AR-expressing PC3 and DU145 prostate tumor cells. By comparison to the integrin-mediated pathways in the parental cells (see Preliminary Results in Project Narrative), the effect of AR expression in these cells can be determined.

**Task B: Determine if LM signaling via AR to Bcl-xL rescues survival in PI-3K inhibited cells.** (Months 5-10).

1. PC3 AR-expressing cells will be treated with or without DHT and screened for total Bcl-xL levels by immunoblotting.
2. PC3 AR-expressing cells will be plated on LM or CL and screened for total Bcl-xL levels by immunoblotting.
3. PC3 AR-expressing cells will be treated with PI-3K inhibitor LY294002 or vehicle, and plated on LM or CL with or without DHT, and the levels of Bcl-xL will be monitored by immunoblotting.
4. Obtain siRNA to Bcl-xL and control scrambled virus.
5. PC3 AR-expressing and vector control cells infected with siRNA against Bcl-xL will be plated on LM or CL in the presence of LY294002 to inhibit PI-3K and cell survival death will be monitored by TUNEL.
6. Stable PC3 cell lines expressing mutant variants of AR will be generated.
7. The ability of the AR mutants to rescue cell death after treatment with PI-3K and adhesion to LM or CL will be monitored by TUNEL staining and FACS analysis.

Outcome: It will be determined if Bcl-xL is the downstream targets of integrin- and AR-mediated survival signaling. It will be determined how AR functions to regulate survival.

**AIM 2: Determine how survival is regulated in normal primary prostate epithelial cells expressing AR *in vitro.***
My *working hypothesis* is that the in primary prostate epithelial cells, expression of AR will shift regulation of survival from being regulated primarily through adhesion via integrins to being regulated through cell-cell adhesion by E-cadherin. Furthermore, autophagy may contribute to cell survival.

**Task A: Determine whether AR expression affects survival signaling pathways in PECs.** (Months 9-25)

1. PECs will be treated with KGF+DHT to induce a double stratified cell layer where AR expression is induced in the upper layer.

2. KGF/DHT-treated cells will be treated with specific pharmacological inhibitors PD168393 or AG1475, PD98059 or U0126, and LY294002.
3. Cell viability will be analyzed by TUNEL staining and confocal microscopy at daily time points up to 96 hours.
4. KGF/DHT-treated cells will be treated with AR specific siRNA and AR levels will be monitored using immunostaining and confocal microscopy. Cell viability will be analyzed by TUNEL staining and confocal microscopy at daily time points up to 96 hours.

Outcome: The requirement of EGFR/Erk and PI-3K for survival in the AR expressing normal cells will be determined.

**Task B: Determine if integrins mediate survival in PECs expressing AR.** (Months 9-12).

1. Integrin expression in both cell layers of KGF/DHT treated PECs will be monitored by immunostaining and confocal microscopy.
2. KGF/DHT-treated cells will be treated with blocking antibodies to 6 integrin. The viability of the AR positive PECs in the upper layer will be assessed by TUNEL staining and confocal microscopy.

Outcome: Changes in integrin expression between the cell layers of the KGF/DHT treated PECs will be determined. The requirement for integrin 6 for survival in the AR positive upper layer will be determined.

**Task C: Determine if E-cadherin signaling to PI-3K and Src is responsible for cell survival in PECs expressing AR.** (Months 25-30).

1. KGF/DHT-treated cells will be treated with Src specific siRNA and the effects on Src levels in the two cell layers will be monitored by immunostaining, confocal microscopy, and immunoblotting.
2. Cell viability after Src inhibition will be analyzed by TUNEL staining and confocal microscopy at daily time points up to 96 hours.
3. KGF/DHT-treated cells will be treated with E-cadherin blocking antibody and cell viability will be analyzed by TUNEL staining and confocal microscopy at daily time points up to 96 hours.
4. The effects on PI-3K and Src signaling will be monitored by immunoblotting using phospho-specific antibodies after blocking E-cadherin.
5. KGF/DHT-treated cells will be made to over-express Src or inhibit Pten expression using virus or siRNA respectively.
6. Cell viability will be analyzed by TUNEL staining and confocal microscopy at daily time points up to 96 hours in Src or Pten manipulated cells when E-cadherin is blocked.

Outcome: It will be determined if Src and E-cadherin function to regulate survival. It will be determined if PI-3K and Src are downstream of E-cadherin. The ability of Src or Pten loss to resuce survival in E-cadherin blocked cells will be determined.

**Task D: Determine if autophagy is mediating survival in PECs expressing AR.** (Months 30-34)

1. KGF/DHT-treated cells will be starved of growth factors and nutrients over a daily time course up to 96 hours. Autophagy will be monitored by immunoblotting for LC3 processing and for the presence of LC3-GFP puncta.
2. Autophagy will be inhibited by the lysosomal inhibitor, Chloroquine or the Vps34 inhibitor 3-methyladenine. The viability of the AR positive PECs in the upper layer will be assessed by TUNEL staining and confocal microscopy.

Outcome: It will be determined if autophagy is occurring in the AR positive upper layer, and if this process is required for survival under starvation conditions.

# E-cadherin-mediated survival of androgen-receptor-expressing secretory prostate epithelial cells derived from a stratified in vitro differentiation model

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## Summary

The androgen receptor (AR) is expressed in differentiated secretory prostate epithelial cells in vivo. However, in the human prostate, it is unclear whether androgens directly promote the survival of secretory cells, or whether secretory cells survive through androgen-dependent signals from the prostate stroma. Biochemical and mechanistic studies have been hampered by inadequate cell-culture models. In particular, large-scale differentiation of prostate epithelial cells in culture has been difficult to achieve. Here, we describe the development of a differentiation system that is amenable to functional and biochemical analysis and its application to deciphering the survival pathways in differentiated AR-expressing epithelial cells. Confluent prostate epithelial cell cultures were treated with keratinocyte growth factor (KGF) and dihydrotestosterone. After 2 weeks, a suprabasal cell layer was formed in which cells no longer expressed α2, α3, α6, αv, β1 or β4 integrins or p63, K5, K14, EGFR, FGFR2IIIb or Bcl-2, but instead expressed AR and androgen-induced differentiation markers, including K18, K19, TMPRSS2, Nkx3.1, PMSA, KLK2 and secreted prostate-specific antigen (PSA). Differentiated prostate cell survival depended on E-cadherin and PI3K, but not KGF, androgen, AR or MAPK. Thus survival of differentiated prostate epithelial cells is mediated by cell-cell adhesion, and not through androgen activity or prostate stroma-derived KGF.

**Key words:** Prostate, Epithelial, Androgen receptor, Secretory cells, Survival, Differentiation

## Introduction

Epithelial cells serve several vital functions. For instance, all epithelial cells act as a barrier to protect organs from external environmental assault, as exemplified by the skin. Intestinal epithelial cells are required for the absorption of nutrients, and mammary and prostate epithelial cells are primarily secretory. Proper regulation of epithelial differentiation is crucial for the development and maintenance of barrier and organ function. Differentiation of epithelial cells has been extensively characterized in the epidermis. The basal layer of the epidermis consists of proliferating keratinocytes that adhere to a basement membrane via integrins. Loss of basal cell adhesion through integrin β1 initiates terminal differentiation, resulting in flattening of the cells, expression of differentiation proteins, and subsequent cornification, which ultimately produces several distinct stratified cell layers that make up the epidermis (Levy et al., 2000; Lippens et al., 2005).

The epithelium of the human prostate consists of two cell layers, a basal layer and a secretory layer. Similar to other stratified epithelium, prostate basal cells are mitotic and adhere to a basement membrane (Knox et al., 1994; Uzgare et al., 2004; van Leenders and Schalken, 2003). Prostate basal cells give rise to terminally differentiated secretory cells (Knox et al., 1994; Uzgare et al., 2004; van Leenders and Schalken, 2003). However, unlike other epithelia, prostate epithelial cell differentiation is regulated by androgen signaling (Berger et al., 2004; Cunha et al., 1987; Heer et al., 2007; Ling et al., 2001; Whitacre et al., 2002). The androgen receptor (AR) is a nuclear transcription factor activated in response to the steroid hormone androgen (Lamb et al., 2001). AR is expressed only in the differentiated secretory cells and not in the basal cells (Lamb et al., 2001). It is unclear exactly how androgen regulates epithelial differentiation. However, tissue combination studies from AR-null mice suggest that androgen stimulation of AR in the early developing mesenchyme, and not the epithelium, is solely responsible for the induction of epithelial morphogenesis in vivo (Cunha et al., 2004).

Androgen also appears to be important for secretory cell survival, in that anti-androgen therapies specifically kill the secretory cells, leaving the basal cells intact (Denis and Griffiths, 2000). Furthermore, restoration of androgens results in regeneration of the secretory cell compartment. However, tissue recombination experiments, as well as studies using conditional knockout mice that lack AR only in prostate epithelium, suggest that AR does not directly regulate epithelial survival (Cunha et al., 2004; Wu et al., 2007). Instead, androgen stimulation of the AR-positive stromal cells of the prostate might induce secreted factors that regulate secretory cell survival. Keratinocyte growth factor (KGF) and FGF10 are two factors secreted by the stromal cells, though not in an androgen-dependent manner (Alarid et al., 1994; Cunha et al., 2004; Sugimura et al., 1996; Thomson, 2001). KGF and FGF10 are both involved in murine prostate organogenesis and can induce differentiation of isolated prostate epithelial cells (Alarid et al., 1994; Cooke et al., 1991; Cunha, 1996; Donjacour et al., 2003; Heer et al., 2006; McKeenhan, 1991; Sugimura et al., 1996). In some cases, KGF can substitute for androgens and it is likely that KGF and AR signaling pathways interact (Thomson et al., 1997). KGF has also been reported to promote differentiation and survival of the epithelium of the skin, lung and eye (Geiger et al., 2005; Marchese et al., 1997; Ray et al., 2003). KGF acts specifically on epithelial



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cells and has been reported to activate p38 MAPK signaling (Heer et al., 2006).

Clarification of the roles of androgen and KGF in prostate epithelial differentiation and survival has been hampered by our inability to culture normal differentiated AR-expressing secretory cells in vitro. Prostate epithelial cells (PECs) cultured from normal human prostate tissue consist primarily of AR-negative basal cells and their transient amplifying derivatives. Previous studies in our lab have demonstrated that survival of cultured PECs is specifically mediated through α3β1-integrin-dependent adhesion (Edick et al., 2007). Similarly, basal keratinocytes are dependent on α3β1 integrin for their survival (Manohar et al., 2004). During keratinocyte differentiation, basal cells lose integrin expression as well as adhesion to matrix as they are extruded to the upper layers of the skin (Watt, 2002). In suprabasal keratinocytes, as well as in other epithelia, cell-cell adhesion structures such as E-cadherin appear to promote survival through phosphoinositide 3-kinase (PI3K) signaling, and when PI3K signaling is lost these cells die (Calautti et al., 2005; Espada et al., 2009; Rivard, 2009). Whether the same survival mechanisms are operative in differentiated secretory prostate epithelial cells is unknown, and the role of KGF or androgen in prostate epithelial cell survival remains unresolved.

In this study, confluent cultured primary prostate basal epithelial cells were induced to differentiate following treatment with KGF and androgen. After 2 weeks, differentiated AR-expressing secretory cells appeared as a secondary cell layer above the basal cells. This model was used to identify the signaling pathways important for prostate secretory cell survival. This new model will serve as a valuable tool for understanding the biology of prostate secretory epithelial cells, a cell population previously not available for extensive analysis.

## Results

### Differentiation of confluent PECs by KGF and DHT

Previous studies have demonstrated that KGF might be an important epithelium differentiation factor in many tissues, including prostate epithelium (Alarid et al., 1994; Cunha, 1996; Heer et al., 2006; Peehl et al., 1996; Sugimura et al., 1996). Androgen, acting via the androgen receptor, also plays an important role in prostate epithelial cell differentiation (Berger et al., 2004; Cunha et al., 1987; Heer et al., 2007; Ling et al., 2001; Whitacre et al., 2002). To determine if the combination of KGF and androgen is sufficient to induce differentiation of prostate cells grown in culture, human primary basal prostate epithelial cells (PECs) grown to confluency in monolayer cell cultures were treated with 10 ng/ml KGF and 5-10 nM androgen (DHT). Culturing the cells for 10-15 days with KGF and DHT resulted in the formation of stratified cell patches consisting of at least two cell layers, resembling the bilayer of basal and secretory cells observed in the prostate epithelium in vivo (Fig. 1A-C).

To determine if the stratified cells expressed differentiation markers specific to prostate secretory cells, expression of AR and the AR-target protein prostate-specific antigen (PSA) were examined by fluorescence confocal microscopy. Cells in a higher z-plane than the bottom cells, stained positive for AR and PSA (Fig. 1B). AR expression was both nuclear and cytoplasmic, whereas the secreted protein PSA had the expected cytoplasmic localization (Fig. 1B). AR expression was uniform throughout the top cells, whereas PSA expression was often concentrated at the upper membrane of the top-most cells, consistent with that of a secreted protein (not shown). Neither AR nor PSA was found in the bottom cells (Fig. 1B). Additionally, the AR-regulated proteins Nkx3.1 and TMPRSS2 (Bowen et al., 2000; Lin et al., 1999a; Murtha et al., 1993; Young et al., 1992) were expressed in top cells and not in bottom cells (Fig. 1C).

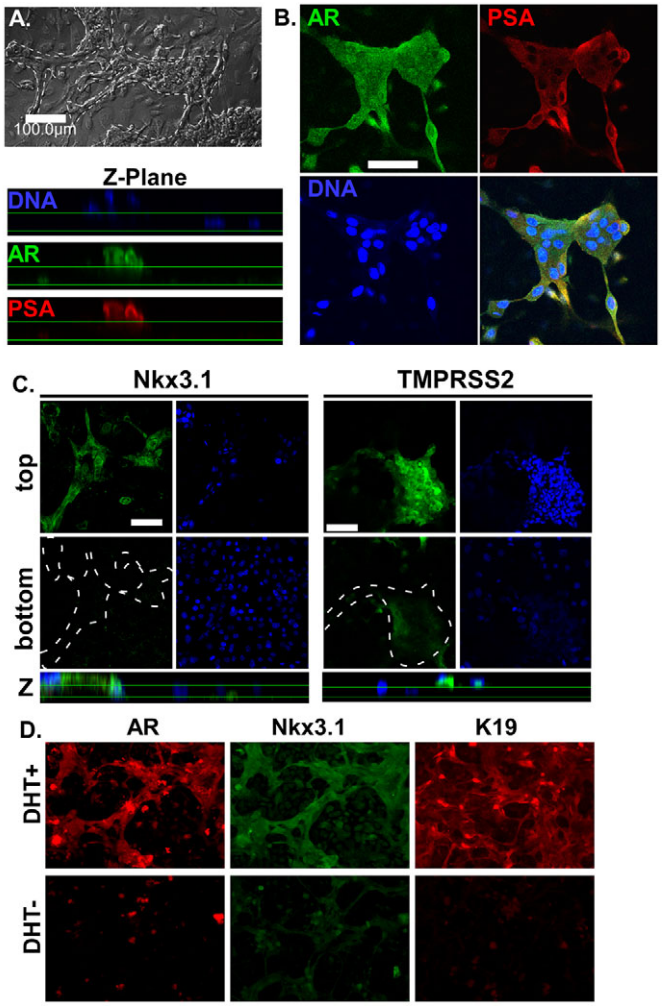


**Fig. 1. AR and AR-dependent proteins are present in the differentiated cultures.** Confluent primary prostate epithelial cells (PECs) were induced to differentiate with 10 ng/ml KGF and 5 nM DHT for 10-14 days. (**A**) DIC image of a differentiated culture shows an upper layer of cells (outlined with dashed white line) on top of a confluent bottom cell layer. Scale bars: 100 µm. (**B**) A 14-day differentiated culture was immunostained for AR (green) and PSA (red). Nuclei (blue) were visualized by Hoechst 33258 staining. (Left panel) A z-section image was compiled from 30 confocal x-y sections representing a thickness of 38.0 µm. Horizontal lines demarcate top and bottom cell layers. (Right panels) Confocal images of top cells in 14-day differentiated cultures. Scale bar: 50 µm. (**C**) Differentiated PEC cultures were immunostained for Nkx3.1 and TMPRSS2 (green) and imaged by confocal microscopy. Nuclei were stained with Hoechst 33258 (blue). Representative top and bottom cells and z-plane images (Z) are shown below. Scale bar: 100 µm. (**D**) PECs were induced to differentiate for 14 days with KGF in the presence or absence of 10 nM DHT. Cells were immunostained with AR, Nkx3.1 and K19 and imaged by epifluorescence microscopy.

To determine the extent to which androgen stimulation contributes to PEC differentiation, PECs were treated for 10-14 days with KGF in the presence or absence of DHT, and the expression of AR, AR-target proteins, and differentiation cell markers was

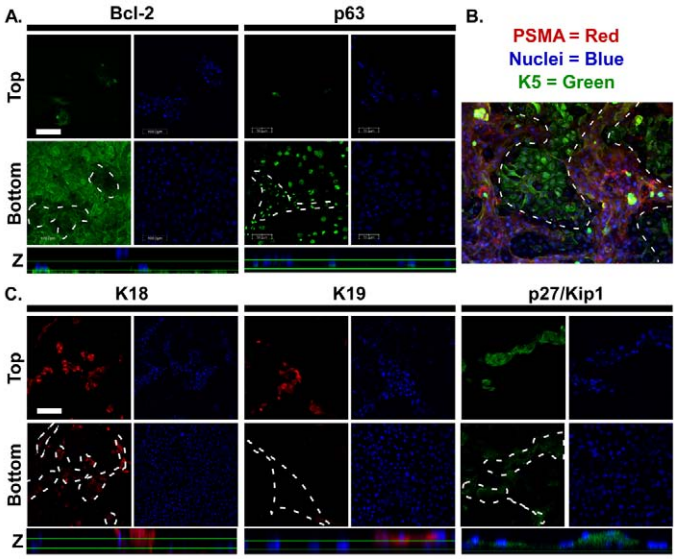


**Fig. 2. Differentiation-specific epithelial markers present in the top cells of differentiated cultures.** 10- to14-day differentiated cultures were immunostained for (**A**) Bcl-2, p63 (green), (**B**) K5 (green), PMSA (red), (**C**) K18, K19 (red), and p27 (Kip1; green) expression, and images were captured by confocal microscopy (A,C) or epifluorescence (B). Nuclei were stained with Hoechst 33258 (blue). Representative top and bottom cells are shown. Representative z-section images (Z) were compiled from 10-15 confocal x-y sections representing a thickness of 17.04 (±3.27) μm. Horizontal lines demarcate top and bottom cells. Scale bars: 100 μm.

monitored. PSA, Nkx3.1 and TMPRSS2 were only expressed when DHT was present (Fig. 1D, PSA and TMPRSS2 not shown). Intriguingly, cytokeratin markers, K18 and K19, were also expressed only in the presence of androgen (Fig. 1D, K18 data not shown). Furthermore, there was a dramatic increase in AR expression itself when DHT was present.

KGF, in the absence of DHT, was sufficient to induce formation of stratified cells, with maximal formation occurring between10 and 15 days. PECs treated with KGF in the presence of KGF-blocking antibody did not stratify. Confluency of the cultures was essential. Subconfluent cells treated with KGF and DHT did not form stratified clusters. KGF-induced stratification occurred equally efficiently, with or without the supplementary bovine pituitary extract (BPE) and EGF in the culture medium. Occasionally, a few small stratified clusters appeared in BPE-containing medium without KGF treatment, suggesting the presence of low levels of KGF and/or an additional unknown factor(s) in BPE that can promote differentiation at a low efficiency. KGF-blocking antibodies prevented the appearance of these occasional clusters. The optimal concentration of KGF was 10 ng/ml. Lower doses (1-5 ng/ml) resulted in fewer clusters and higher doses (20-50 ng/ml) did not generate more clusters. DHT alone was not sufficient to induce stratification. DHT plus KGF treatment dramatically increased the number of top cells seen after 15 days. DHT was required for expression of androgen-dependent markers in the top cells. FGF10, a functionally related FGF family member shown to be important for prostate development in vivo (Donjacour et al., 2003; Igarashi et al., 1998), could also induce PEC differentiation in the presence of DHT. Differentiation was reproducibly observed in cells derived from two different patients at three different passage numbers (passages 2, 3 and 4). It was observed however, that once cells reached passage 5, the efficiency of differentiation was dramatically reduced. Furthermore, we were able to induce differentiation in an immortalized cell line derived from a third patient. We observed that these more proliferative immortalized cultures took a few days longer to reach maximal differentiation.

### Stratified cells express additional differentiation markers

Markers specific to basal and differentiated epithelial cells populations were examined in the stratified cultures. The basal markers Bcl-2, K5 and K14 (McDonnell et al., 1992; Wang et al., 2001) were expressed predominantly in the bottom cells; occasionally a few K5- and K14-positive cells were seen in the top cells (Fig. 2A,B, K14 not shown). Basal marker p63 (Parsons et al., 2001; Signoretti et al., 2000) was associated only with bottom cells (Fig. 2A). EGFR, which is predominately expressed in basal cells (Sherwood and Lee, 1995), was associated primarily with bottom cells (not shown). Epithelial cell markers K19 and PMSA were expressed only in the top cells and not in the bottom cells (Fig. 2B,C). K18, as well as the cell cycle inhibitor p27 (Kip1) (Peehl et al., 1994; Tsihlias et al., 1998; Wernert et al., 1987; Yang et al., 1998), was expressed predominately in the top cells (Fig. 2C).

### Differentiation induces integrin loss

Consistent with previous observations of differentiating epithelium in vitro and in vivo (Gustafson et al., 2006; Heer et al., 2006; Levy et al., 2000; Li et al., 2008), epifluorescence and confocal imaging revealed that the subpopulation of the cells undergoing differentiation lost expression of many integrins, including α2, α3, α6, β1 and β4 (Fig. 3A,B). Basal cells also expressed αv-, but not β3- or β5-integrin subunits. None of these integrins were present in the differentiated cells (not shown). Cultured PECs secrete and organize a laminin 5 (LM5)-rich matrix (Yu et al., 2004); the differentiating cell population that lost integrin expression also no longer produced LM5 (Fig. 3A,B). Although it appears, by confocal imaging, that the cells directly below the top cells do not express integrin or LM5, it is possible that there is incomplete antibody penetrance into the lower cells. To address this, a timecourse study was performed. We observed a decrease in LM5 expression as early as 3 days after KGF and DHT treatment and a complete loss after 8 days. At 8 days decreased β1 integrin expression was observed in LM5-negative cells prior to formation of the second cell layer (supplementary material Fig. S1A). Therefore, cells directly underneath the top layer also lose LM5 and integrin expression. LM5 loss might be the trigger that initiates differentiation.

### Differentiated cells respond to androgen

AR expression could be detected by immunoblotting of cell lysates from whole cultures treated with KGF and DHT (Fig. 4A). Expression of the androgen-dependent secreted proteins, KLK2 and PSA, was monitored in differentiated cultures by RT-PCR. KLK2 and PSA mRNAs were present only when DHT was present in the culture (Fig. 4B). Furthermore, secreted PSA, up to 0.8 ng/ml, could be detected by ELISA (Fig. 4C). PSA secretion required androgen and increased with increasing DHT concentration. The expression and secretion of an androgen-regulated protein in an androgen-dependent manner indicates the presence of differentiated prostate secretory cells in the culture, and that AR is functional and regulates expression of differentiation markers.

Overall, this in vitro differentiation model recapitulates many aspects of in vivo differentiation as assessed by the specific markers



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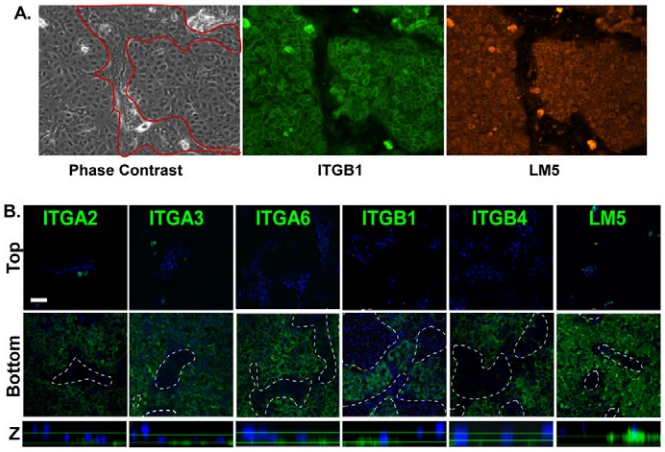


**Fig. 3. Prostate epithelial differentiation is accompanied by loss of integrin expression.** (**A**) Integrin β1 (ITGB1; green) and laminin 5 (LM5; red) expression in 10-day differentiated cultures were monitored by DIC (left panel) and epifluorescence microscopy following immunostaining. (**B**) 14-day differentiated cultures were immunostained to detect expression (green) of integrins α2 (ITGA2), α3 (ITGA3), α6 (ITGA6), β1 (ITGB1), β4 (ITGB4) and laminin 5 (LM5) and imaged by confocal microscopy. Nuclei (blue) were visualized by Hoechst 33258 staining. Representative confocal x-y sections of the top and bottom cells are shown. The area located directly beneath the top layer of differentiated cells is indicated with dashed white lines in the bottom image. Representative z-section images (Z) were compiled from 10-15 confocal x-y sections representing a thickness of 17.04 (±3.27) μm. Horizontal lines demarcate top and bottom cells.

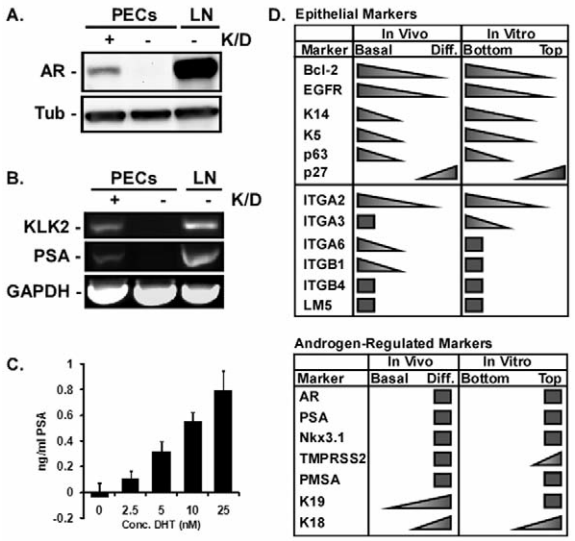


**Fig. 4. Differentiated cells respond to androgen.** (**A**) Immunoblot for AR expression in cultures of PECs treated with or without KGF and DHT(K/D) for 16 days. LNCaP cells (LN) were used as a positive control for AR expression. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. (**B**) Levels of KLK2 and PSA mRNA isolated from 14-day differentiated (K/D) cultures were analyzed by PCR and compared to LNCaP (LN) cells. GAPDH served as a control. (**C**) Levels of secreted PSA from 14-day differentiated cultures treated with KGF and increasing amounts of DHT were determined by ELISA. (**D**) Summary of epithelial and androgen-dependent markers observed and their relative expression in the lower (bottom) and upper (top) cells. Expression observed in vitro is compared with that reported in vivo.

(Fig. 4D). In addition to the induction of markers common to most differentiating epithelial cells, the presence of DHT markedly stimulates the expression of markers unique to prostate secretory epithelial cells. Hereafter when referring to this model, the AR-expressing top cells will be referred to as secretory-like cells and the AR-negative bottom cells as basal cells.

### Isolation of secretory-like cells

Treatment of differentiated cultures with dissociation buffer preferentially dislodges the secretory-like cells. FACS analysis indicates that 96.6% (±0.8%) of the isolated dislodged population is negative for cell surface α6 integrin, whereas 97.19% (±1.70%) of the cells not dislodged are positive for α6 integrin (Fig. 5A). Further FACS sorting based on surface staining of α6 integrin and TMPRSS2 revealed that on average 87.92% (±3.71%) of the α6-integrin-negative cells were positive for TMPRSS2. A representative example is provided in Fig. 5B. Immunoblotting of separated cells indicated that some remaining basal cells expressed AR as well as full-length TMPRSS2 protein; however, only the secretory-like cells expressed the cleaved and activated form of TMPRSS2 (Fig. 5C) (Wilson et al., 2005). Conversely, only the basal cells expressed Bcl-2 and EGFR, whereas K5 was predominately found in the basal cells (Fig. 5D).

### Secretory cell survival is dependent on PI3K and E-cadherin, but not KGF or androgen

In previous studies, we demonstrated that integrin-mediated activation of EGFR and downstream signaling to ERK, but not PI3K signaling, is required for the survival of basal PECs (Edick et al., 2007). However, the differentiated secretory-like PECs have lost integrin expression, no longer adhere to the LM5 matrix, and have significantly lower levels of EGFR, suggesting that other survival pathways must be important for secretory cell survival. It has been suggested that secretory cell survival might be dependent on stromal-derived growth factors, including KGF (Kurita et al., 2001). One possibility is that the KGF used to induce differentiation, might also be necessary for survival. To test this, the KGF receptor FGFR2IIIb (Giri et al., 1999) mRNA levels were analyzed in the isolated secretory-like cells and basal cells by RT-PCR. Only the basal cells expressed FGFR2IIIb mRNA (Fig. 6A). Furthermore, removal of KGF after 15 days of differentiation did not induce cell death (not shown). Thus it is unlikely that KGF is regulating cell survival in the secretory-like cells.

Dissociated secretory-like cells and the remaining basal cells were screened for ERK and AKT activation by immunoblotting. Active ERK was present only in the basal cells, but not in the secretory-like cells (Fig. 6B). Activated AKT was present in both types of cells (Fig. 6C). Thus, ERK signaling probably does not regulate survival in differentiated cells, whereas the PI3K pathway could. Since the differentiated cells remain adherent to the bottom basal cells, we also investigated whether there is an increase in expression of the cell-cell adhesion molecule E-cadherin in the secretory-like cells. Compared with the basal cells, E-cadherin levels were elevated in the secretory cell population that also does not express α6β1 integrin (Fig. 6D). E-cadherin can lead to activation of PI3K signaling in skin and colonic epithelium as well as in some tumor cell lines (Calautti et al., 2005; Hofmann et al., 2007; Pang et al., 2005). Blocking antibodies to E-cadherin

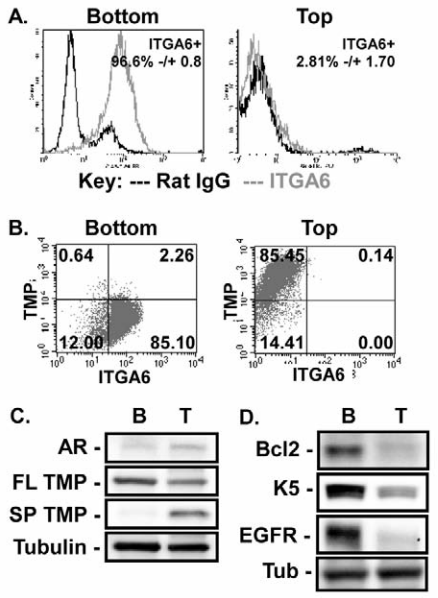


**Fig. 5. Isolation of secretory-like cells.** (**A**) Following treatment of 14-day cultures with dissociation buffer the separated upper (Top) and lower (Bottom) cells were subjected to FACS to measure cell surface α6-integrin expression (gray line) versus control antibody (black line). Dead cells were excluded using PI staining. Values are the percentage α6-integrin-positive cells. (**B**) Cells isolated and sorted for α6 integrin as in A were further sorted based on TMPRSS2 (TMP) surface expression. Values are the percentage of positive cells in each quadrant. Data is from one typical experiment. (**C**,**D**) Bottom (B) and top (T) cells, obtained after treatment with dissociation buffer, were analyzed by immunoblotting for AR and TMPRSS2 (C), and Bcl-2, K5 and EGFR (D) expression. Tubulin immunoblotting served as a loading control.

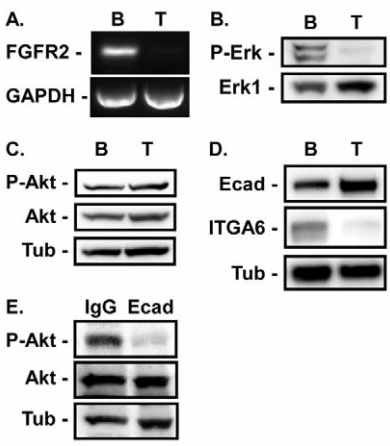


**Fig. 6. Signaling pathways in secretory-like cells.** Following treatment of 14-day cultures with dissociation buffer, mRNA or protein was isolated from the lower (B) and upper cells (T). (**A**) Levels of FGFR2IIIb mRNA (FGFR2) were assessed by PCR. GAPDH served as a control. (**B**) Levels of ERK activation (P-Erk) in the lower and upper cells were monitored by immunoblotting of cell lysates with phosphospecific ERK antibodies. Total levels of ERK and tubulin in the lysates were measured by immunoblotting. (**C**) Levels of AKT activation (P-Akt) in the lower and upper cells were monitored by immunoblotting of cell lysates with phosphospecific AKT antibodies. Total levels of AKT and tubulin in the lysates were measured by immunoblotting. (**D**) Levels of E-cadherin (Ecad) and α6 integrin (ITGA6) in the lower and upper cells were analyzed by immunoblotting. Tubulin immunoblots served as loading controls. (**E**) 14-day cultures were treated with control IgG or E-cadherin-blocking antibody (Ecad) for3 hours. Levels of AKT activation (P-Akt) in the isolated upper cells were monitored by immunoblotting of cell lysates with phosphospecific AKT antibodies. Total levels of AKT and tubulin in the lysates were measured by immunoblotting.

suppressed AKT activity in both the secretory-like (Fig. 6E) and the basal cells (not shown).

The relative importance of the different signaling pathways on secretory-like cell survival was investigated. Fourteen-day KGF and DHT-differentiated cultures were placed in KGF- and DHT-free basal medium without any pituitary extract or EGF supplement for 72 hours to reduce any signaling induced by the growth medium (Fig. 7A). Visually, the starved cell cultures appeared viable, and the upper secretory-like cell layer remained intact (data not shown). Then the starved differentiated cultures were treated with specific inhibitors in the presence or absence of freshly added DHT or KGF and analyzed over a 72-hour timecourse. Cell death was measured in the upper secretory-like cell layer by immunostaining for active caspase 3/7, TUNEL staining or propidium iodide (PI) uptake. Staining was quantified as described in Materials and Methods. Inhibition of PI3K signaling with LY294002 resulted in maximal secretory-like cell death at 72 hours, where 60% of the cells stained positive for PI (Fig. 7B). Furthermore, inhibition of PI3K, but not EGFR, induced a 7.0- to 7.5-fold increase in secretory cell caspase 3 activity (Fig. 7C), and a 5.5- to 5.7-fold increase in TUNEL staining (Fig. 7D; supplementary material Fig. S1B). Maximal annexin V staining was observed 66 hours after LY294002 treatment (not shown). Secretory-like cell survival was not dependent on DHT or KGF, and addition of DHT or KGF was unable to promote cell survival in the absence of PI3K signaling (Fig. 7B-D). Although KGF should not be present in the media, and prostate epithelial cells have been reported not to produce KGF, KGF-blocking antibodies were used to prevent any endogenous or remaining KGF from promoting cell survival. KGF-blocking antibodies had no effect on cell survival (data not shown). KGF has been reported to activate p38, and Jnk can promote survival during stress (Heer et al., 2006; Leppä and Bohmann, 1999; Mehta et al., 2001). Inhibiting p38 with SB202190, JNK with 420119, or ERK with PD98059 did not result in cell death, suggesting these pathways are not critical for secretory cell survival (supplementary material Fig. S1C). The lack of effect of the inhibitors on cell survival was not due to a failure to inhibit signaling, as the concentrations of drugs used here did effectively block signaling to their specific targets in basal cells.

Cell-cell adhesion via E-cadherin was inhibited by treatment of differentiated cells with two different preparations (lots) of E-cadherin-blocking antibodies. Inhibition of cell-cell adhesion with one lot of E-cadherin-blocking antibody resulted in maximal cell death at 48 hours with over 80% of the cells staining positive for PI (Fig. 7E). By 66 hours, no secretory-like cells remained in the cultures. A second lot of E-cadherin antibody resulted in a seven- to eightfold increase in TUNEL staining 72 hours after treatment (Fig. 7F). The presence of DHT or KGF could not protect cells from death due to loss of E-cadherin function. No cell death was observed in the lower basal cells. Furthermore, blocking E-cadherin lead to a decrease in AKT activation (see Fig. 6E), indicating that cell-cell adhesion mediated by E-cadherin promotes secretory-like cell survival through PI3K signaling.



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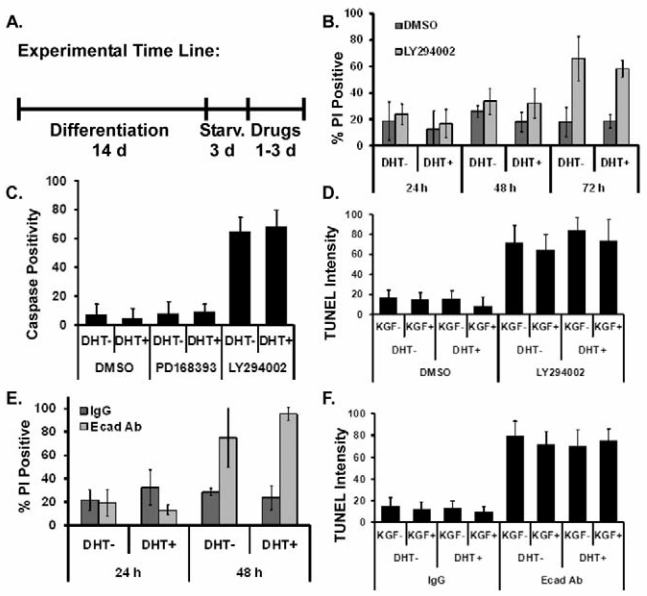


**Fig. 7. Secretory-like cells are dependent on PI3K and E-cadherin, but not androgen or KGF, for survival.** (**A**) PECs were treated with KGF and DHT for 14 days (Differentiation), then starved of growth factors and DHT for 3 days (Starv.), and then treated with pharmacological inhibitors (Drugs) for 1-3 days. In some cases DHT or KGF were also added back with the inhibitors. (**B**) Differentiated cultures were treated with vehicle (DMSO) or PI3K inhibitor LY492004 in the presence or absence of DHT for 24, 48 or 72 hours. Cell viability in the top cells was measured by quantifying the number of cells with high PI staining and expressed as percentage PI-positive cells. (**C**,**D**) Differentiated cultures were treated with vehicle (DMSO), EGFR inhibitor PD168393, or PI3K inhibitor LY492004 in the presence or absence of DHT or KGF. After 72 hours cell viability in the top cells was assessed using (C) cleaved caspase-3 or (D) TUNEL staining. Total DNA was stained with PI. Six fields per experiment and condition were examined and positive pixels counted using the software program Imagine as outlined in the Material and Methods. TUNEL- or cleaved caspase-3-positive pixels were normalized to the total number of stained DNA pixels in the region of interest and expressed as relative intensity of caspase-3 or TUNEL staining. Error bars indicate standard deviation. *n*=3. (**E**) Differentiated cultures were treated with non-specific mouse IgG (IgG) or with E-cadherin-blocking antibody (Ecad Ab; lot 2) in the presence or absence of DHT for 24 or 48 hours. Cell viability was measured by PI staining. (**F**) Cell viability of differentiated cultures treated with non-specific mouse IgG (IgG) or with E-cadherin-blocking antibody (Ecad Ab; lot 1) in the presence or absence of DHT or KGF for 72 hours was measured by TUNEL staining.

Although DHT was not important for survival of the differentiated secretory-like cells, it is theoretically possible that AR, acting via an androgen-independent mechanism might still be important for cell survival. To address this, 14-day KGF- and DHT-differentiated cultures were transfected with an AR-specific siRNA pool or a scrambled siRNA sequence. Confocal imaging of the transfected cells 72 hours later demonstrated the absence of AR expression in the upper cells (Fig. 8A). Absence of AR expression also resulted in loss of androgen-dependent cell markers such as Nkx3.1 and K19 (Fig. 8A). Cell viability of the AR siRNA-treated cells was assessed by TUNEL staining. Loss of AR had no effect on secretory-like cell viability (Fig. 8B). Thus, AR and androgen signaling are not required to maintain the viability of differentiated secretory-like cells derived from our in vitro culture system.

## Discussion

By treating cultured primary prostate basal epithelial cells with androgen and KGF, we have established an in vitro differentiation model of the prostate epithelium. The differentiated cells in our culture system possess the important features of terminally differentiated secretory prostate epithelial cells in vivo: they do not proliferate, they adhere to a basal cell layer and not to the basement membrane, they express AR protein, and they respond to DHT by inducing AR-dependent genes. Specifically, the cells express androgen-sensitive proteins, such as KLK2, PSA, Nkx3.1, PMSA and TMPRSS2. In addition, cleaved TMPRSS2 is present in the upper, but not the lower cells and PSA is secreted into the culture medium. Furthermore, cytokeratin K18 and K19 expression was found to be dependent on androgen. K18 expression has previously been reported to be regulated by androgen (Heer et al., 2007; Ling et al., 2001), and K19 has been suggested to be responsive to estrogen (Choi et al., 2000); however, both K18 and K19 promoters lack classical androgen response elements, making the mechanism of regulation unclear.

Further evidence for terminal differentiation is that the cells did not revert to basal cells when isolated and re-plated, and they failed to reattach, probably because of continued loss of integrin and/or matrix expression. Furthermore, after 21-25 days in culture the upper cells sloughed off and a few activated caspase-3-positive cells were seen in the aging cultures (data not shown), similar to what is observed in vivo. Oddly, no more differentiated cells reappeared. Only about 20% of the cells appeared to be capable of undergoing differentiation, suggesting that the differentiated cells are derived from a distinct subpopulation of basal cells. The lack of continued differentiation after 25 days may indicate depletion of these special cells and a lack of ability to renew. The population of differentiation-competent cells is not likely to be stem cells, since 20% of the cells are capable of undergoing differentiation. However, we cannot rule out the possibility that these cells arose from some stem cell-like progenitor within the culture. Further analysis would be required to determine if the progenitors are analogous to the Nkx3.1-positive luminal stem cell recently described (Wang et al., 2009). However, whatever the progenitor, it apparently cannot renew in the context of our culture conditions.

Although many aspects of the differentiated cells recapitulate what is observed in vivo, there still remain some differences. For instance, the distribution of AR demonstrates a significant amount of cytoplasmic expression in the in vitro culture system, whereas in vivo AR is primarily nuclear. Another difference is the absence of columnar cells. In addition, a few K5- and/or K14-positive cells were sometimes seen in the upper layer, which has also been reported in another differentiation model (van Leenders et al., 2000). Hence, we cannot unequivocally say whether our secretory-like cells represent completely terminally differentiated prostate cells and there are still some distinctive morphological differences between our cultures and what is seen in the prostate gland in vivo.

Other studies have reported on prostate epithelial differentiation in vitro. Although these studies were informative, they were limited since AR and AR-regulated proteins were not expressed (Dalrymple et al., 2005; Danielpour, 1999; Garraway et al., 2003; Gu et al., 2006; Gustafson et al., 2006; Yasunaga et al., 2001). A few studies have reported seeing stratified layering similar to ours after treating prostate epithelial cells in vitro with retinoic acid, FGF and/or insulin (Gustafson et al., 2006; Peehl et al., 1994; Robinson et al., 1998; van Leenders et al., 2000); however, in these models the top layer of cells either failed to express AR or still expressed basal markers.

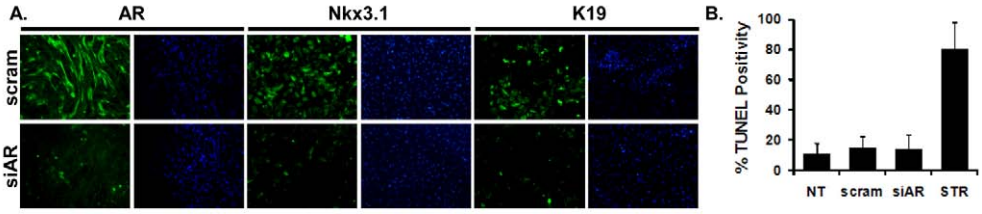


**Fig. 8. AR is not required for secretory-like cell survival.** PECs were treated with KGF and DHT for 14 days and then transfected with scrambled siRNA (scram) or AR-specific siRNA (siAR) to block AR expression. (**A**) Cells were immunostained with AR, Nkx3.1 and K19 (green) and imaged by epifluorescence microscopy. Nuclei were stained with Hoechst 33258 (blue). (**B**) Differentiated cultures left untreated (NT) or treated with scrambled siRNA (scram), AR siRNA (siAR), or staurosporine (STR) were measured for cell viability by TUNEL staining.

In our model, the top secretory-like cells expressed AR and lost basal marker expression. In one case, gland-like buds and extensions were observed to form from confluent cell cultures, reminiscent of acini structures in overall shape but without lumens (van Leenders et al., 2000). We have also observed cases where cells appear to form mounds. By confocal imaging, some of them appear to have formed a hollow mound (data not shown). A recent study demonstrated that co-treatment of prostate basal cells with the monoamine oxidase A inhibitor clorgyline, 1,25-dihydroxyvitamin $D_3$, all-trans retinoic acid and TGF-β1 induced AR expression and loss of basal marker K14 (Zhao et al., 2008), suggesting that there might be alternative mechanisms to inducing prostate epithelial cell differentiation.

In contrast to other published systems, we have demonstrated that our model can be utilized for biochemical and genetic manipulation. It is amenable to treatment with pharmacological inhibitors or siRNA to study signaling and biological pathways. Furthermore, exploitation of differential cell surface markers and adhesion properties can be used to separate basal from secretory-like cells to separately analyze RNA and protein expression.

It is unknown whether AR represses integrin expression or whether loss of integrin expression must precede expression of AR. Unpublished data from our laboratory and others demonstrates that re-expression of AR in prostate cancer cell lines results in decreased integrin expression (Bonaccorsi et al., 2000; Nagakawa et al., 2004). However, in our model we observed that not all integrin-negative cells were AR positive, suggesting that integrin loss might precede AR expression. Furthermore, LM5 matrix loss preceded integrin loss, which preceded stratification and robust AR expression in our timecourse studies. Heer et al. have demonstrated that blocking integrin β1 is sufficient to induce partial differentiation; however, cells do not reach terminal differentiation since the cells do not express AR-regulated genes (Heer et al., 2006). This suggests that loss of adhesion can initiate early differentiation and may even be required, but that integrin loss alone is not sufficient for terminal differentiation. By contrast, unbound integrin β1 is sufficient to initiate terminal differentiation in keratinocytes (Levy et al., 2000; Watt, 2002). In mammary epithelium, however, loss of integrin β1 suppresses differentiation (Naylor et al., 2005).

Interestingly, in most of the reported prostate differentiation models (including ours), confluent cultures were necessary for stratification. In addition, previous studies suggest that cell cycle inhibition is a prerequisite for expression of secretory cell markers K18, K19 and AR (Danielpour, 1999; Garraway et al., 2003; Gustafson et al., 2006; Litvinov et al., 2006). We similarly saw a loss in cell proliferation in the differentiating cell population (data not shown). This led us to develop the following model for prostate differentiation (Fig. 9). Basal cells are proliferative and a subset begins to undergo growth arrest once the cells are confluent. Treatment with KGF causes a select population of cells, perhaps those that express higher levels of the KGF receptor FGFR2IIIb (Giri et al., 1999), to lose LM5 and then integrin expression, causing the cells to detach. Integrin loss and detachment might then trigger low AR expression. AR expression was not detectable by immunostaining in cultures treated with only KGF, in which integrin expression was lost; however, some AR expression was detectable in the basal cells from the differentiated cultures by immunoblotting. The presence of androgen in the culture appears to be necessary to allow the integrin-deficient cells to express AR at a higher level, which then turns on AR-dependent differentiation-specific genes.

Work by Heer et al. suggests that AR might be expressed at low levels in primary prostate epithelial cells and is rapidly degraded by the proteosome (Heer et al., 2007); hence androgen treatment might stabilize and/or help drive production of AR protein. In fact AR mRNA has been detected in some cultured prostate epithelial cells (Litvinov et al., 2006). However, in our studies and those of others, androgen alone is not very effective in inducing AR expression (Litvinov et al., 2006). Thus, additional events are required to induce stable AR expression even in the presence of androgen. Reduced cell proliferation caused by strong growth suppression or loss of cell adhesion, which is also growth suppressive, might be necessary. Significant increases in AR

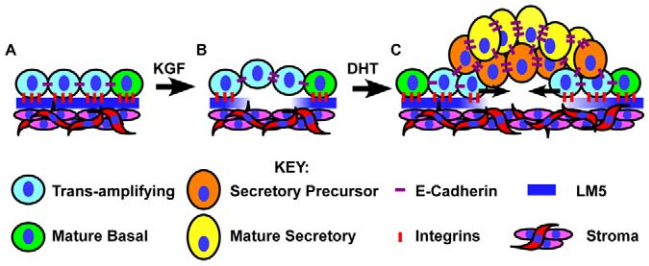


**Fig. 9. Model of differentiation.** (**A**) Confluent primary prostate basal cells secret and adhere to a matrix rich in LM5 via integrins, which physically separates the epithelial cells from the stromal cells. (**B**) After treatment with KGF, a sub-population of transient amplifying cells loses expression of LM5 and subsequently integrins, resulting in loss of adhesion. Concurrently, there is increased cell-cell adhesion via E-cadherin. (**C**) Secretory-like precursor cells arise in concert with androgen treatment, which induces their differentiation into mature secretory cells. Transient amplifying cells at the edge continue to proliferate to fill in the space generated by detachment of cells and movement into the top layer. After 10-14 days, cells become stratified as more transient amplifying cells are committed to terminal differentiation.



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expression can be detected in isolated suspended cells in the presence of androgen (Heer et al., 2007), thus supporting cell detachment as a potential mechanism required for stabilizing AR.

Previous work from our laboratory has demonstrated that integrin-mediated survival of primary prostate basal cells requires integrin-induced EGFR signaling to ERK, but not PI3K signaling (Edick et al., 2007). In this study we have expanded our analysis of survival mechanisms to secretory-like prostate epithelial cells and demonstrated that secretory-like cells depend on a non-integrin-dependent mechanism for cell survival that involves cell-cell interactions through E-cadherin. Interestingly, there is switch from ERK-dependent survival in the basal cells to PI3K-dependent survival in the secretory-like cells. In the secretory-like cells EGFR levels dropped dramatically and EGFR-dependent signaling to PI3K was not required for survival (blocking EGFR had no effect on secretory cell survival). Interestingly, in prostate cancer, there appears to be a strong dependence on PI3K signaling for survival, as these cells tend to acquire mutations in Pten, a negative regulator of PI3K signaling (Bertram et al., 2006; Edick et al., 2007; Lin et al., 1999b; Wen et al., 2000). This suggests that prostate cancer might arise from a more differentiated cell that has already acquired dependence on PI3K for its survival.

In our studies, secretory cell survival was not dependent on the presence of androgen, and knockdown of AR with siRNA in differentiated cells did not induce their death. The lack of dependence on androgen or AR for secretory cell survival in our human culture system is in agreement with genetic and tissue recombination studies in mice. Conditional knockout of AR in mature mouse prostates results in decreased numbers of secretory cells without inducing cell death, suggesting that AR functions to increase secretory cell numbers by promoting differentiation rather than cell survival in mature glands (Wu et al., 2007). Tissue recombination experiments using mesenchyme and epithelium from AR-negative or wild-type mice demonstrate that AR expression in the epithelium is not required for early prostate development, indirectly ruling out a role for AR in epithelial cell survival in newly formed glands (Cunha et al., 2004). Thus, in both models, as well as ours, androgen is responsible for the synthesis of secretory proteins and the secretory function of the prostate.

If androgen and AR do not act cell autonomously to control epithelial cell survival, then why do only the AR-expressing epithelial cells die upon castration-induced androgen deprivation (Evans and Chandler, 1987; Mirosevich et al., 1999)? One possibility is that AR signaling in the stromal cells promotes survival by paracrine factors that act on the epithelial cells (Verhoeven and Swinnen, 1999). In our model the paracrine function of KGF, known to be expressed by stromal cells in vivo, was required for differentiation; however, it was dispensable for cell survival in committed differentiated cells. Thus, the nature of the paracrine survival factor(s) remains undetermined. In our in vitro model, survival was highly dependent on E-cadherin-based cell-cell adhesion and signaling to PI3K. Whether paracrine factors in vivo are responsible for maintaining survival via E-cadherin or whether they act on other pathways remains to be determined.

Our study supports a simpler concept that the role of stromal-derived paracrine factors is to act primarily on the stem and/or basal cells, whose proliferation and regenerative capacity is driven by these factors. As terminally differentiated cells are sloughed into the lumen, basal cells are triggered to proliferate and differentiate to replace the lost cells. Under androgen-ablative conditions, the loss of paracrine factors in the stroma prevents stem cell and/or basal cell renewal and the terminally differentiated cells eventually slough off and are not replaced. Re-administration of androgen restores basal cell proliferation and differentiation, and subsequent restoration of secretory cells. This model would preclude the need for stromal factors acting directly on the secretory cells.

An alternative model to explain castration-induced loss of prostate secretory cells involves the observation that castration reduces blood flow and microvasculature collapse in the gland, inducing a state of hypoxia (Buttyan et al., 2000). It would appear that secretory cells are much more sensitive to such stress than the basal or stromal cells. This might be related to a lack of extracellular matrix support that provides additional survival signaling cues to the basal and stromal cells. Alternatively, hypoxia might affect the production of the paracrine factors required for maintenance of epithelial differentiation or survival.

In summary, we have established an in vitro differentiation model of human prostate epithelium composed of stratified cells that recapitulates many in vivo characteristics of basal and secretory cells, including AR-dependent differentiation and function. This model can be treated with pharmacological inhibitors and siRNA to study biochemical and genetic effects and the differentiated secretory-like cells can be isolated for further analysis. We have further established that although KGF, AR and androgen are important for initiating the differentiation process and AR is important to maintain the androgen-dependent phenotype of secretory-like cells, these factors are not required for survival of the committed differentiated cells. The primary critical mechanism driving cell survival is E-cadherin-based cell-cell adhesion and subsequent activation of the PI3K signaling pathway.

## Materials and Methods

### Cell culture

Human primary prostate epithelial cells (PECs) derived from prostectomy specimens were isolated, cultured, and verified to be free of stromal contamination as described previously (Edick et al., 2007; Gmyrek et al., 2001). Specific patient samples used in this study were again verified to be negative for the stromal cell marker smooth muscle actin by immunostaining. PECs were grown in keratinocyte-SFM medium (Invitrogen) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF). Experiments were reproducibly performed in cells derived from two different patients at three different passage numbers (passage 2, 3 and 4). In addition, at least three separate primary cultures from each patient were used. Experiments were verified at least three times for each of the two patients. We were also able to induce differentiation in an immortalized cell line derived from a third patient. The AR-positive prostate cancer cell line LNCaP was purchased from ATCC. LNCaP cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 IU penicillin, 50 μg/ml streptomycin, 0.225% glucose, 10 mM HEPES, and 1 mM sodium pyruvate.

### Differentiation assay

To induce differentiation, a 10-cm culture dish of confluent PECs was divided equally between three eight-chambered slides (Lab-Tek). Cells were grown in keratinocyte-SFM supplemented with BPE, EGF, 10 ng/ml keratinocyte growth factor (KGF; Calbiochem), and 5-10 nM dihydrotestosterone (DHT; Sigma) for 10-18 days. KGF and DHT were replenished three and five times a week, respectively. For larger-scale experiments, three 10-cm plates of confluent PECs were combined onto one 10-cm dish and treated with KGF and DHT for 21-30 days.

### KGF blocking experiments

KGF-FGF7 blocking antibody (clone 29522) was purchased from R&D Systems. 2 μg/ml KGF blocking antibody or IgG control was added immediately prior to KGF addition. Differentiation of PECs was then assessed by immunofluorescent staining for differentiation markers.

### Cell surface integrin and TMPRSS2 expression analysis

Whole cultures of differentiated PEC cultures were placed in suspension by washing the cells twice with PBS, treating with cell dissociation buffer (Gibco, Invitrogen) for 5 minutes, then adding TrypLE Express trypsin (Gibco, Invitrogen). Cells were then washed with wash buffer (1% sodium azide, 2% FBS-PBS) and incubated with primary antibodies or control IgG molecules for 1 hour at 4°C. Cells were washed twice and incubated with fluorescently labeled secondary antibodies for 1 hour at

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4°C in the dark. Cells were washed twice more, and fluorescence was detected using a Becton-Dickinson FACSCalibur four-color flow cytometer with CellQUEST Pro Software v5.2.1 (Becton-Dickinson).

### Isolation of differentiated cells

Differentiated PEC cultures were washed with 1 mM EDTA in PBS without calcium or magnesium, and then incubated for 5 minutes with 1 mM EDTA-PBS. Cells were then incubated with cell dissociation buffer (Gibco, Invitrogen) for 6-8 minutes. The top layer of cells could then be removed by pipetting the cell dissociation buffer over the cells; the bottom confluent cell layer remained attached to the culture vessel. The isolated cells were used directly or undifferentiated α6-integrin-expressing cells were separated from the differentiated cells using α6 integrin antibodies and FACS as described above using fluorescently conjugated integrin α6 antibody (BD Pharmingen). Cells were sorted on a Becton-Dickinson FACSAria special order system 12-color flow cytometer using FACSDiVa software v5.2 (Becton-Dickinson).

### Immunoblotting

Total cell lysates were prepared for immunoblotting as previously described (Edick et al., 2007; Miranti, 2002). Briefly, cells were lysed with Triton X-100 lysis buffer and 45-75 μg of total cell lysates in 2× SDS sample buffer were boiled for 10 minutes. Samples were run on SDS polyacrylamide gels following standard SDS-PAGE protocols and transferred to PVDF membrane. Membranes were blocked in 5% BSA in TBST for 2 hours at room temperature, then were probed with primary antibody overnight at 4°C. Membranes were washed three times, and incubated with horseradish peroxide-conjugated secondary antibodies (Bio-Rad) in 5% BSA in TBST for 1 hour at room temperature. After washing an additional three times, signals were visualized using a chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

### Immunoblotting antibodies

Antibodies for phospho-specific AKT (S473) or phospho-specific ERK1/2 (T202/Y204) were purchased from Cell Signaling. Antibodies for total ERK were from Becton-Dickinson Transduction Labs and total AKT antibodies have been described previously (Bill et al., 2004). α6 integrin and TMPRSS2 antibody were gifts from Anne Cress (University of Arizona, Phoenix, AZ) and Peter Nelson (Fred Hutchinson Cancer Research Institute, Seattle, WA) (Lucas et al., 2008), respectively. Androgen receptor antibody (441) was purchased from Santa Cruz Biotechnology. E-cadherin antibody (clone HECD1) was purchased from Zymed. Tubulin antibody (clone DM1A) was purchased from Sigma.

### Immunofluorescence

Differentiated PEC cultures were fixed with 4% paraformaldehyde (Mallinckrodt Chemicals) for 10 minutes and permeabilized for 4 minutes with 0.2% Triton X-100 (EMD) at room temperature. Cells were then blocked with 10% normal goat serum (Pierce) for 2 hours at room temperature before incubation with primary antibodies overnight at 4°C. Cells were incubated with appropriate secondary antibodies for 1 hour at room temperature. DNA was visualized by staining with Hoechst 33258 (Sigma) for 10 minutes at room temperature. Cells were washed three times with PBS between all steps. Coverslips were mounted on the slides using Gel-Mount (Biomeda).

Specific antibodies against proteins of interest were obtained as indicated in supplementary material Table S1 and used for immunofluorescent (IF) staining at the stated dilutions. Whole IgG antibodies for controls were purchased from Pierce. Species appropriate Alexa Fluor 488 or 546 antibodies (Molecular Probes, Invitrogen) were used as secondary antibodies for indirect fluorescence.

### Microscopy

Epifluorescence images were acquired using a Nikon Eclipse TE300 fluorescence microscope with OpenLab v5.5.0 image analysis software (Improvision). Confocal images were acquired by sequential detection using a Zeiss 510 Meta NLO v4.2, or Olympus FluoView 1000 LSM using FluoView software v5.0.

### PSA quantification

Differentiated PEC cultures in eight-chambered slides were grown in the presence or absence of DHT for 72 hours in 200 μl per well of growth medium. To quantify PSA concentrations in conditioned medium, a human PSA ELISA kit (Abzyme) was used according to the manufacturer's directions with the following modifications: the entire 200 μl samples were incubated 50 μl at a time per well for 1 hour each. PSA standards were added to coated wells during the final 50 μl of sample incubation.

### Reverse transcription PCR (RT-PCR) for differentiation markers

Human KLK2, human KLK3 (PSA), FGFR2IIIb and GAPDH mRNA levels were quantified in differentiated cells by RT-PCR. Total RNA was isolated from upper and lower cell populations of dissociated cells from differentiated cultures or from LNCaP cells using TRIzol (Gibco) and chloroform (Sigma-Aldrich). Contaminating DNA was then removed using a RNAse-free DNAse kit (Qiagen) following manufacturer's directions. RT-PCR was performed on 1-2 μg RNA with the primers listed in supplementary material Table S2 using the One-Step RT-PCR kit (Qiagen) following manufacturer's directions. RT-PCR products were analyzed on a 2% agarose-TBE gel and DNA was visualized with ethidium bromide and a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

### Small interfering RNA transfections

A pool of four small interfering RNAs (siRNA) against androgen receptor (siGENOME SMARTpool) or a non-targeting sequence were purchased from Dharmacon. Differentiated cultures were transfected with 20 nM siRNA in keratinocyte-SFM medium using siLentFect lipid reagent (Bio-Rad) and Opti-MEM (Invitrogen) medium following manufacturer's directions. The medium was changed 16 hours after transfection.

### Cell survival assays

Differentiated PECs were starved of growth factor in keratinocyte-SFM medium containing no supplements, KGF, or DHT for 72 hours. Then DMSO (control; Sigma), pharmacological inhibitors 0.5 μM PD168393, 2 μM LY294002, 20 μM PD90859, 10 μM SB209102, 10 μM 420119 (all purchased from Calbiochem), 1 μM staurosporine (Promega) or 1 μg/ml E-cadherin-blocking antibody (SHE78-7, Calbiochem) or non-specific mouse IgG (Sigma) was added; in some experiments, siRNAs were used to knock down AR expression (Dharmacon). Cells were incubated for 24, 48, 66 or 72 hours after drug, antibody or siRNA addition. LY294002 was replenished 48 hours after its initial addition. To assess cell viability, cells were fixed and DNA fragmentation was monitored using terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) following the protocol of the APO-BrdU TUNEL Assay Kit (BD Pharmingen). On several occasions, cleaved caspase 3 (Asp175) staining with antibody clone 5A1 from Cell Signaling was also used to measure cell viability of fixed cells. TUNEL and caspase activity were quantified using Imagine software (Qian et al., 2006). Total TUNEL- or caspase-positive pixels were normalized to total propidium iodide-stained DNA pixels in fixed cells and expressed as relative intensity of TUNEL staining. This quantification is based on pixel counts and does not necessarily reflect the percentage of positive cells, but rather the relative intensity of TUNEL or caspase 3 staining between treated and untreated cultures. As an alternative method for measuring cell viability, unfixed cells were treated with propidium iodide (PI). High intensity PI staining of dead, i.e. permeabilized cells, was quantified on a per cell basis and expressed as the percentage PI-positive cells.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/2/266/DC1

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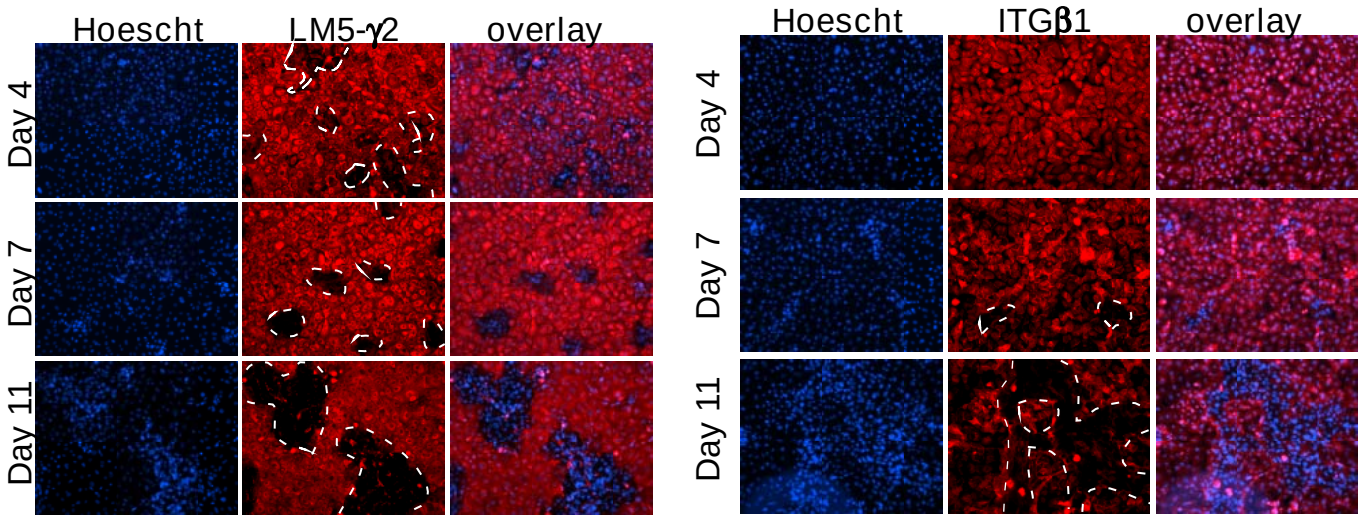
A.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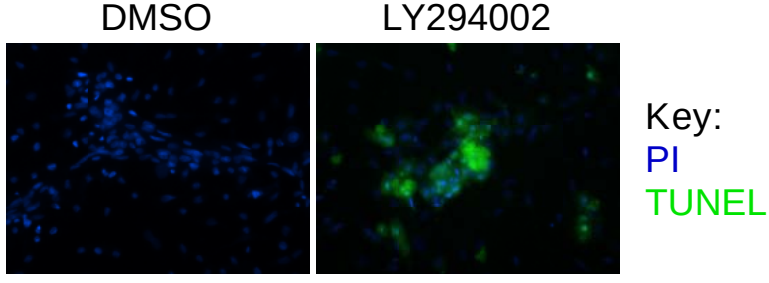
LM5-γ2
overlay
Day 4
Day 7
Day 11
Hoescht
ITGβ1
overlay
Day 4
Day 7
Day 11

B.


DMSO
LY294002
Key:
PI
TUNEL

C.

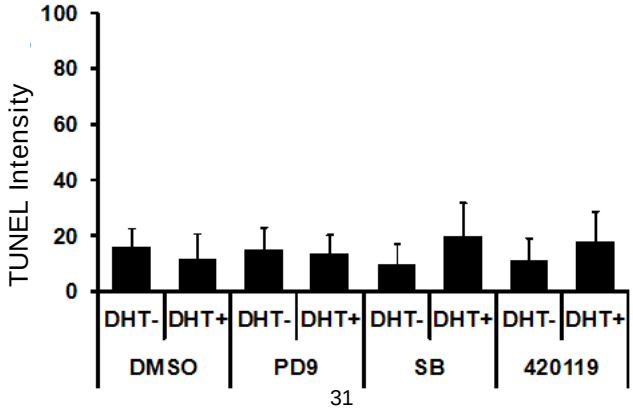

TUNEL Intensity
100
80
60
40
20
0
DHT-
DHT+
DHT-
DHT+
DHT-
DHT+
DHT-
DHT+
DMSO
PD9
SB
420119

**Table S1. Immunofluorescent antibodies**

| Protein | Clone | IF dilution | Company |
|---|---|---|---|
| goatAb PSA | C-19 | 1:100 | Santa Cruz Biotechnology |
| mAb AR | 411 | 1:500 | Santa Cruz Biotechnology |
| mAb Bcl-2 | 100 | 1:50 | Santa Cruz Biotechnology |
| mAb E-cadherin | HECD-1 | 1:100 | Zymed/Invitrogen |
| mAb EGFR | Ab12 | 1:200 | Neomarkers |
| mAb ITGA2 | P1H5-E9 | 1:10 | Gift from W.G. Carter |
| mAb ITGA2 | PIE6-1-1 | 1:10 | Gift from W.G. Carter |
| mAb ITGA3 | P1F2-1-1 | 1:10 | Gift from W.G. Carter |
| mAbITGAV | 272-17E6 | 1:250 | AbCam |
| mAb K18 | CY-90 | 1:100 | Sigma |
| mAb K19 | A53-BIA2 | 1:50 | Sigma |
| mAb LM5 ($\gamma$2 chain) | D4B5 | 1:100 | Chemicon |
| mAb p63 | 4A4 | 1:100 | Santa Cruz Biotechnology |
| mAb PSA | 18127 | 1:100 | R&D Systems |
| mAb PSMA | YPSMA-1 | 1:250 | AbCam |
| mAb SMA | 1A4 | 1:100 | Zymed/Invitrogen |
| mAbTMPRSS2 | P5H9-A3 | 1:250 | Gift from P.S. Nelson |
| rAb K5 | AF138 | 1:500 | Convance |
| rAb Ki67 | | 1:200 | Zymed/Invitrogen |
| rAb Kip1/p27 | G173-324 | 1:100 | Pharmigen |
| rAb Nkx3.1 | H-50 | 1:500 | Santa Cruz Biotechnology |
| ratAb ITGA6 | GoH3 | 1:100 | BD Pharmingen |
| ratAb ITGB1 | AIIB2 | 1:100 | Iowa State Univ. Hybridoma Bank |
| ratAb ITGB4 | P4GH-1 | 1:10 | Gift from W.G. Carter |

## Table S2. RT-PCR primers

| Target | Fwd Primer (5′→3′) | Rev Primer (5′→3′) | Ref |
|---|---|---|---|
| ITGA6 | GCTGGTTATAATCCTTCAATATCAATTGT | TTGGGCTCAGAACCTTGGTTT | (Tapia et al., 2008) |
| ITGB1 | GTGGTTGCTGGAATTGTTCTTATT | TTTTCCCTCATACTTCGGATTGAC | (Tapia et al., 2008) |
| AR | TTTTCAATGAGTACCGCATGC | TCTCGCAATAGGCTGCACG | (Heer et al., 2007) |
| FGFR2IIIb | ATTGTTCTCCTGTGTCTG | CTTTTCAGCTTCTATATCC | (Mehta et al., 2000) |
| GAPDH | ACCACAGTCCATGCCATCAC | TCCACCACCCTGTTGCTGTA | (Sun et al., 2008) |
| KLK2 | GGCAGGTGGCTGTGTACAGTC | CAACATGAACTCTGTCACCTTCTC | (Shaw and Diamandis, 2007) |
| KLK3 (PSA) | CCCACTGCATCAGGAACAAAAGCG | GGTGCTCAGGGGTTGGCCAC | (Shaw and Diamandis, 2007) |

# REPORT OF INVENTIONS AND SUBCONTRACTS

*(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)*

*Form Approved*
*OMB No. 9000-0095*
*Expires Jan 31, 2008*

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to the Department of Defense, Executive Services Directorate (9000-0095). Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THE ABOVE ORGANIZATION. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

| 1.a. NAME OF CONTRACTOR/SUBCONTRACTOR | c. CONTRACT NUMBER | 2.a. NAME OF GOVERNMENT PRIME CONTRACTOR | c. CONTRACT NUMBER | 3. TYPE OF REPORT *(X one)* |
|---|---|---|---|---|
| Van Andel Research Institute | W81XWH-08-1-0058 | USAMRAA | Same as 1.c. | X a. INTERIM ☐ b. FINAL |

| b. ADDRESS *(Include ZIP Code)* | d. AWARD DATE *(YYYYMMDD)* | b. ADDRESS *(Include ZIP Code)* | d. AWARD DATE *(YYYYMMDD)* | 4. REPORTING PERIOD *(YYYYMMDD)* |
|---|---|---|---|---|
| 333 Bostwick Ave NE<br>Grand Rapids, MI 49503 | 20080101 | 820 Chandler Street<br>Fort Detrick, MD 21702-5014 | 20080101 | a. FROM 20090101<br>b. TO 20091231 |

## SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

| NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a. | TITLE OF INVENTION(S) b. | DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c. | ELECTION TO FILE PATENT APPLICATIONS *(X)* d. | | | | CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e. | |
|---|---|---|---|---|---|---|---|---|
| | | | (1) UNITED STATES | | (2) FOREIGN | | | |
| | | | (a) YES | (b) NO | (a) YES | (b) NO | (a) YES | (b) NO |
| Miranti, Cynthia K., and Lamb, Laura E. | A Method for Inducing Differentiation of Androgen Receptor-Expressing Prostate Epithelial Cells | 61/173,783.<br>(provisional) | X | | | X | | X |

| f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR | | g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED | |
|---|---|---|---|
| (1) (a) NAME OF INVENTOR *(Last, First, Middle Initial)*<br>Knudsen, Beatrice | (2) (a) NAME OF INVENTOR *(Last, First, Middle Initial)* | (1) TITLE OF INVENTION | (2) FOREIGN COUNTRIES OF PATENT APPLICATION |
| (b) NAME OF EMPLOYER<br>Fred Hutchinson Cancer Research Center | (b) NAME OF EMPLOYER | | |
| (c) ADDRESS OF EMPLOYER *(Include ZIP Code)*<br>1100 Fairview Avenue North, M5-A864<br>Seattle, Washington 98109 | (c) ADDRESS OF EMPLOYER *(Include ZIP Code)* | | |

## SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

| NAME OF SUBCONTRACTOR(S) a. | ADDRESS *(Include ZIP Code)* b. | SUBCONTRACT NUMBER(S) c. | FAR "PATENT RIGHTS" d. | | DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e. | SUBCONTRACT DATES *(YYYYMMDD)* f. | |
|---|---|---|---|---|---|---|---|
| | | | (1) CLAUSE NUMBER | (2) DATE *(YYYYMM)* | | (1) AWARD | (2) ESTIMATED COMPLETION |
| None | | | | | | | |

## SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* ☐ SMALL BUSINESS or X NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

| a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)* | b. TITLE | c. SIGNATURE | d. DATE SIGNED |
|---|---|---|---|
| | | | |